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Identification of cancer vulnerabilities using focused chemical and genetic screening approaches

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Submitted by

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Declaration

The work described in this thesis was carried out at the Christian Doppler Laboratory for Chemical Epigenetics and Antiinfectives at the Research Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, the Medical University of Vienna, as well as the General Hospital of Vienna. Permissions for reprint of figures were obtained from the respective publishers. This doctoral thesis was written by Bernhard (hereinafter referred to as "Bernd") Boidol. Contributions to individual experiments and writing are described separately for Chapter 2.1 and Chapter 2.2 below.

Contributions Chapter 2.1 (partially reprinted with permission from (Boidol *et al*, 2017))

Bernd Boidol and Christoph Kornauth performed the experiments; Emiel van der Kouwe, Nicole Prutsch, Lukas Kazianka, Sinan Gültekin, Julia Koller, Anna Ringler, Bernadette Hilgarth, Marius E. Mayerhoefer, Michael Panny, Olaf Merkel, and Lukas Kenner organized clinical samples and data; Gregor Hoermann, Georg Hopfinger, Marie-Bernadette Aretin, Peter Valent, Ingrid Simonitsch-Klupp, Richard Moriggl, Olaf Merkel, Lukas Kenner, and Ulrich Jäger provided reagents and intellectual contributions; Alexander Hauswirth, Wolfgang R. Sperr, Ulrich Jäger, and Philipp B. Staber were responsible for patient treatment and ethical guidelines; Stefan Kubicek and Philipp B. Staber designed and oversaw the study; **Bernd Boidol**, Christoph Kornauth, Stefan Kubicek, and Philipp B. Staber analyzed the data; and **Bernd Boidol** and Philipp B. Staber wrote the manuscript.

Contributions Chapter 2.2

Bernd Boidol performed experiments and analyzed data unless stated otherwise below; Sandra Schick prepared ATAC-seq samples; André Rendeiro performed ATAC-seq data analysis; Anna Ringler prepared drug screening plates and readouts; next-generation DNA sequencing, RNA-seq library preparation and sequencing, ATAC-seq sample sequencing, as well as raw data preparation was performed by Thomas Penz and Michael Schuster at the Biomedical Sequencing Facility (BSF) at CeMM; Guido Boehmelt and Marc Petronczki provided intellectual input; **Bernd Boidol** and Stefan Kubicek analyzed genomic sequencing data; Stefan Kubicek compiled the epigenome-focused shRNA library; Stefan Kubicek designed and oversaw the study; and **Bernd Boidol** wrote the chapter.

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Abstract

The research conducted during the past decades has generated a tremendous stock of biological knowledge regarding the molecular processes that govern cancer development and progression. Many of these findings have contributed to an armamentarium of drugs and techniques that exploit the inherent vulnerabilities of cancers. Still, the majority of cancer entities are incurable and oftentimes the high expectations that were built by the advent of new technologies or novel findings in cancer research were not met, dampening the hopes of scientists, physicians, and patients alike. The reasons underlying the challenges in drug target identification and drug development are diverse. For one, the rarity of certain forms of cancer makes it difficult to unravel the pathophysiology behind the disease and drug screening efforts are often hampered due to the limited resources available in these instances. Despite the low frequency of each of the approximately 200 rare cancers, they represent up to a non-negligible 22% of all cancer cases in total. On the other hand, many of the most frequently mutated genes in cancer patients have been identified but it is becoming clear that these mutations are often difficult to target directly, such as inactivating mutations in tumor suppressor genes for example. Novel ways of tackling these challenges are urgently sought after. We focused on one cytologically defined rare cancer, T-cell prolymphocytic leukemia, and a group of genetically defined common cancers harboring mutations in the BAF chromatin remodeling complex. For neither of the two, targeted treatment options are currently available.

In order to identify new vulnerabilities in T-cell prolymphocytic leukemia (T-PLL), a rare and very aggressive T-lymphoid neoplasm, we employed a library of well-annotated, clinically approved drugs and small molecules in clinical development to screen a cohort of patients with hematologic malignancy. We identified the BCL-2 inhibitor Venetoclax as specifically active in T-PLL biopsies as well as in T-PLL patients. We could detect an increase in BCL-2 and BCL-X_L expression upon Venetoclax treatment, serving a potential explanation for the development of resistance mechanisms. Moreover, we show that additional drugs in combinations with Venetoclax have synergistic effects *ex vivo*, pointing towards new potential combination treatments in a clinical setting.

Furthermore, we identified a novel vulnerability in the context of BAF chromatin remodeler mutations. The BAF complex is a *bona fide* tumor suppressor with mutation rates of almost 20% across all human cancers. To identify synthetic lethal candidate genes, we performed an epigenome-focused lentiviral RNAi screen using a cell line panel deficient for *ARID1A* and *SMARCA4*, the genes encoding the most frequently mutated subunits of the BAF complex. Validation experiments confirmed the histone chaperone complex CAF-1 subunits as synthetic lethal targets in *ARID1A* mutated cells. Moreover, knockdown of these genes

resulted in a reversal of the ARID1A loss phenotype on a transcriptional and chromatin accessibility level and a drug screen revealed testosterone and 19-nortestosterone as compounds that synergize with the ARID1A-specific cell depletion upon CAF-1 knockdown.

Taken together, the vulnerabilities presented in this thesis allow deeper insight into the biology behind T-PLL and BAF deficient cancers, while at the same time providing a foundation for the future generation of personalized treatment option.

Zusammenfassung

Die Forschungsergebnisse der vergangenen Jahrzehnte haben zu einem gewaltigen Fundus an biologischem Wissen über die molekularen Prozesse der Krebsentstehung und dessen Verlauf geführt. Viele dieser Resultate trugen dazu bei, ein Instrumentarium an Medikamenten und Methoden zu entwickeln, mit denen die Schwachstellen von Krebserkrankungen ausgenutzt werden können. Dennoch sind die meisten Krebsarten noch nicht heilbar und oft wurden die hohen Erwartungen, welche durch das Aufkommen neuer Technologien und Forschungsresultate erzeugt wurden, nicht erfüllt – ein Dämpfer für die Hoffnungen vieler Wissenschaftler, Ärzte, und Patienten zugleich. Die Gründe für die Herausforderungen in der Medikamentenentwicklung und bei der Identifizierung von Zielproteinen sind vielfältig. Zum einen macht die Seltenheit mancher Krebsarten es schwer, die pathologischen Ursachen welche hinter der Erkrankung stehen zu entschlüsseln und Wirkstoff-Screenings werden oft durch die limitierten Ressourcen behindert. Trotz der geringen Häufigkeit jeder einzelnen dieser seltenen Krebsarten stellen sie dennoch 22% aller Krebserkrankungen dar. Zum anderen wurde die Mehrzahl der am häufigsten mutierten Gene in Krebspatienten bereits identifiziert und es zeigt sich immer mehr, dass diese Mutationen häufig schwer direkt angreifbar sind, wie es das Beispiel der inaktivierenden Mutationen in Tumorsuppressorgenen zeigt. Nach einer neuen Art und Weise, wie man diese Herausforderungen bewältigen kann, wird dringend gesucht. Wir haben uns auf eine zytologisch definierte seltene Krebserkrankung, T-Prolymphozyten-Leukämie, und auf eine Gruppe von genetisch charakterisierten Krebsarten mit Mutationen im BAF Chromatin Remodeling Proteinkomplex fokussiert. Eine zielgerichtete, nachhaltige Behandlungsstrategie existiert momentan für keine der beiden Krankheitsklassen.

Um neue Schwachstellen in der T-Prolymphozyten-Leukämie (T-PLL), einer seltenen und sehr aggressiven T-lymphozytischen Tumorerkrankung, zu identifizieren, haben wir eine gut charakterisierte Medikamentenbibliothek benutzt, welche sowohl klinisch zugelassene Medikamente, als auch Wirkstoffe die sich noch in klinischen Entwicklungsphasen befinden beinhaltet. Mit dieser Bibliothek wurden Proben von Patienten mit hämatologischen Erkrankungen gescreent. Wir konnten feststellen, dass der spezifische BCL-2 Inhibitor Venetoclax besonders aktiv in Proben von T-PLL Patienten war, sowie auch in den Patienten selbst. Ebenso konnten wir einen Expressionsanstieg von BCL-2 und BCL-X_L detektieren, welcher als mögliche Erklärung eines Resistenzmechanismus dienen kann. Schließlich zeigen wir, dass verschiedene andere Medikamente synergistische Effekte in Verbindung mit Venetoclax zeigen und auf neue Möglichkeiten der klinischen Kombinationstherapie hindeuten.

Des Weiteren haben wir Angreifbarkeiten bei Mutationen des BAF Chromatin Remodeller identifiziert. Der BAF Proteinkomplex ist ein *bona fide* Tumorsuppressor mit Mutationsraten von fast 20%, gemittelt über allen Krebsarten die den Menschen betreffen. Um synthetisch letale Kandidatengene zu identifizieren haben wir einen auf das Epigenom fokussierten, lentiviralen RNAi Screen durchgeführt. Die dafür benutzten Zelllinien wiesen Mutationen in den Genen *ARID1A* und *SMARCA4* auf, welche die am häufigsten mutierten BAF-Untereinheiten kodieren. In den darauffolgenden Validierungsrunden konnten wir zeigen, dass der Histon-Chaperonkomplex CAF-1 ein synthetisch letales Protein in Zellen ist, deren *ARID1A* Gen Defekte aufweist. Darüber hinaus kehrte der Knockdown von CAF-1 den Phänotyp des *ARID1A*-Defekts um, sowohl auf Transkriptionsebene wie auch in Bezug auf die Zugänglichkeit des Chromatins selbst. Ein zusätzlicher Medikamentenscreen konnte Testosteron und 19-Nortestosterone als Synergiepartner in der CAF-1-vermittelten ARID1A-Zellreduktion identifizieren.

Zusammenfassend erlauben die onkologischen Angreifbarkeiten, welche in dieser Dissertation präsentiert werden, einen tieferen Einblick in die Biologie der T-Prolymphozyten-Leukämie und bei Zellmodellen mit BAF-Defekten. Gleichzeitig stellen sie ein Fundament dar, auf dem man zukünftig personalisierte Behandlungsstrategien aufbauen kann.

Publications arising from this thesis

Parts of chapter 2.1 of this thesis were published in the journal *Blood* in 2017 (Impact factor 2016: 13.164)

First-in-human response of BCL-2 inhibitor Venetoclax in T-cell prolymphocytic leukemia

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Abbreviations

ABC	ATP-binding cassette transporter
ABL	Abelson murine leukemia viral oncogene homolog 1
ACTB	Actin beta
ACTL6A	Actin-like protein 6A
ADP	Adenosine diphosphate
AGO2	Protein argonaute-2
AKT	Protein kinase B alpha
APAF1	Apoptotic peptidase activating factor 1
AR	Androgen receptor
ARID1	AT-rich interactive domain-containing protein 1A
ATAC	Assay for transposase accessible chromatin
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and Rad3-related protein
b2-MG	Beta-2-microglobulin
BAF	BRG1- or HBRM-associated factors
BAX	BCL-2 associated X
BC	Before Christ
BCL-2	B-cell lymphoma 2
BCL-X_L	BCL-2-like protein 1
BCOR	BCL-6 corepressor
BCR	Breakpoint cluster region protein
BH3	BCL-2 homology domain 3
BID	BH3 interacting-domain death agonist
BIM	BCL-2 interacting mediator of cell death
bp	Base pairs
BRCA	Breast cancer gene
BRG1	Brahma protein-like 1
BRM	Brahma homolog
CAF-1	Chromatin assembly factor 1
CCLE	Cancer cell line encyclopedia
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CHAF	Chromatin assembly factor

CHD	Chromodomain-helicase-DNA binding
CHECK2	Checkpoint kinase 2
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisolone
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
COSMIC	Catalogue of somatic mutations in cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
DEG	Differentially expressed gene
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DPF1	Double PHD fingers 1
DSB	Double strand break
ED	Glutamic acid, aspartic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMEM	Eagle's minimum essential medium
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EWS	Ewing sarcoma breakpoint region 1
EZH2	Enhancer of zeste homolog 2
F-12K	Kaighn's modification of Ham's F-12 medium
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FAS	FAS cell surface death receptor
FBS	Fetal bovine serum
FBXW10	F-Box and WD repeat domain containing 10
FDA	Food and drug administration
FLI	Friend leukemia integration 1 transcription factor
FL	Follicular lymphoma
GATA1	Erythroid transcription factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H3K9	Histone H3 lysine 9

HDAC6	Histone deacetylase 6
HeLa	Cell line derived from a cervical carcinoma of Henrietta Lacks
HER-2	Human epidermal growth factor receptor 2
HP1	Heterochromatin binding protein 1
HRR	Homologous recombination repair
HSCT	Hematopoietic stem cell transplantation
IC_{50}	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IgH Eμ	Immunoglobulin H intronic enhancer Eμ
IL2RG	Interleukin 2 receptor subunit gamma
INI1	Integrase interactor 1
INO80	Inositol requiring 80
ISWI	Imitation switch
JAK	Janus kinase
kDa	Kilo Dalton
KER	Lysine, glutamic acid, and arginine
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia sequence 1
Mi-2/NuRD	Nucleosome remodeling Deacetylase
MLL1	Myeloid/lymphoid or mixed-lineage leukemia 1
mM	Millimolar
MOI	Multiplicity of Infection
MOMP	Mitochondrial outer membrane polymerization
mRNA	Messenger ribonucleic acid
MRT	Malignant rhabdoid tumor
mSWI/SNF	Mammalian switch/sucrose non-fermenting
MTCP-1	Mature T-cell proliferation 1
MYC	Avian myelocytomatosis viral oncogene homolog
NCI	National cancer institute
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PARP1	Poly [ADP-ribose] polymerase 1
PBAF	Polybromo-associated BAF
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
PEST	Proline, glutamic acid, serine, threonine
PHF10	PHD finger protein 10
PI3K	Phosphoinositide 3-kinase
PIK3IP1	Phosphoinositide 3-kinase interacting protein 1
PIP	Proliferating cell nuclear antigen interacting peptide regions
PRC2	Polycomb repressive complex 2
PUMA	P53-upregulated modulator of apoptosis
R&D	Research and development
RAS	Rat sarcoma oncogene
RB	Retinoblastoma protein
RbAp48	Retinoblastoma-binding protein p48
RIPA	Radioimmunprecipitation assay buffer
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNAseq	Ribonucleic acid sequencing
RSV	Rous' sarcoma virus
SETDB1	SET domain bifurcated 1
shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SL	Synthetic lethality
SMAC	Second mitochondrial-derived activator of caspases
SMARC	SWI/SNF related matrix associated actin dependent regulator of chromatin
SNF5	Sucrose non-fermenting yeast homolog-like 1
STAT	Signal transducers and activators of transcription
SUZ12	Suppressor of zeste 12 protein homolog
SWI/SNF	Switch/Sucrose non-fermenting
T-PLL	T-cell prolymphocytic leukemia
TBS-T	Tris-buffered saline with Tween20
TCL1	T-cell leukemia 1
TET2	Tet methylcytosine dioxygenase 2
TGF-b	Transforming growth factor beta
THF	Tetrahydrofolate
TNFR1	Tumor necrosis factor receptor 1
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand 1

TSG	Tumor suppressor gene
v-src	V-src avian Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
VEGF	Vascular endothelial growth factor
WD repeat	Beta-transducin repeat
µM	Micromolar

Acknowledgments

A PhD is a journey. I have heard and read this statement many times before I embarked on my own PhD studies but only realized its full validity when I was already on the road. Similar to a PhD, every journey presents challenges that are part of the experience. Challenges that can be joyful to work on and challenges that are tough to master. No matter what challenges the journey poses, each one constitutes a step forward in one's personal development. And at the end of the journey one can look back at the emotions, experiences, surprises, and obstacles encountered on the way, and be grateful for what each single one of them has taught.

Still, a journey is only as good as the people one gets to know while on the trip. During my own journey, I got to know many different people who helped me, taught me, challenged me and laughed with me. I want to mention some of them in particular and thank them for their continuous support.

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1. Introduction

Finding the right drug for the right patient is one of the maxims of biomedical research. It has redefined drug discovery and development efforts and shifted our understanding of disease treatment from a compartmentalized perspective, i.e. defined by pathological criteria and applied as a “one-size-fits-all” approach, to a more holistic view. This drug-patient relationship, which is central to the era of precision medicine, first and foremost requires the identification of molecular targets against which drugs can be developed. The wide field of cancer with its oftentimes heterogeneous genetic background and, as a result, rapid selection and promotion of resistance mechanisms, particularly demands an increasing number of drug targets and target-specific molecules to fulfill the right-drug-right-patient postulation. In the light of new technologies such as next-generation sequencing, CRISPR-mediated (clustered regularly interspaced short palindromic repeats) genome editing, highly sensitive proteomic approaches and high-throughput screening techniques, our understanding of cancer entities and their underlying molecular constitution is becoming more thorough. The identification of genetic aberrations that are common to certain cancer entities has resulted in the advent of blockbuster drugs with high effectiveness against key drivers of malignant transformation and tolerable side effects.

Nevertheless, the first wave of enthusiasm regarding target-based drug development has been dampened by arising challenges. For instance, cases where no obvious common druggable genetic denominator can be identified - be it due to the rarity of disease entities and the concomitantly low statistical power of genetic studies, or the implication of mutations in tumor suppressors rather than oncogenes, which are much more difficult to tackle with small molecules – impose limitations on drug development. The “low-hanging fruits” of drug development have mostly been harvested and the emerging level of disease complexity and dynamics make it arduous to continue with classical drug development approaches. Overcoming these obstacles using new ways of target identification is therefore essential to refine the field of precision medicine, which, at its core, means matching the right drug to the right patient.

The following chapters are going to describe the history of personalized cancer therapy, recent advances in targeted therapy, its challenges, and opportunities to overcome limitations of drug target identification and cancer drug discovery. Furthermore, two examples for which the current models of target identification have not yet met the expectations will be further elaborated.

1.1 From early chemotherapy to personalized medicine

1.1.1 Paul Ehrlich's "magic bullet" concept

At the core of every cancer therapy, be it cytotoxic chemotherapy, targeted therapy, radiation therapy or surgery, lies the basic assumption that malignant cells are distinct from benign cells and that this distinction can be harnessed to treat cancer. The ability to identify and visualize such differences traces back to the successful era of histology in the 19th century. The study of microscopic tissue structures and the advent of novel staining techniques made it possible to differentiate cell types and localize their cellular compartments in different tissue sections. Many of the dyes and techniques discovered in this period are still in use today, such as the hematoxylin and eosin staining technique that was described in 1875 - 1878 as a method to stain cell nuclei and cytoplasm (Titford, 2005; Coleman, 2006; Musumeci, 2014).

Paul Ehrlich, a German physician and scientist born in the second half of the 19th century, used these staining techniques to lay the foundation of chemotherapy and targeted cancer treatment. In his doctoral thesis "Contributions to the Theory and Practice of Histological Staining", Ehrlich describes his discovery of certain types of leukocytes that contained granules which he could stain employing an aniline dye. He named these cells "mast cells" according to his believe that the granulated cells served as feeder cells for the surrounding tissue ("Mast" being the German for fattening of animals) and later, with the help of additional dyes, defined the different types of granulocytes based on their degree of granulation (Crivellato *et al*, 2003; Kay, 2016).

Inspired by contemporary scientists such as Robert Koch and Emil Behring, Ehrlich transitioned his research to the field of immunology where he started to investigate the immunization of live animals with toxins, the development of anti-diphtheria sera, and the innate and adaptive immune system. The work evolved into the formulation of his "side-chain theory" which hypothesized the existence of certain receptors, either on the cell surface or in the blood, that can be bound by antigens which would, in turn, interfere with the function of these very receptors like a "key-and-lock-model". Together with John Newport Langley, Ehrlich later refined his side-chain theory and postulated the existence of "chemoreceptors" located on the cellular surface which would specifically recognize antigens such as toxins (Strebhardt & Ullrich, 2008; Sepkowitz, 2011; Valent *et al*, 2016).

His observation that dyes have a specific affinity for certain cellular structures and cell types in combination with his theories about the chemoreceptors as the reason behind this molecular preference made him start the endeavor of selecting toxic chemicals to target microorganisms and other cells. Under his supervision, a multitude of arsenic compounds was systematically screened in animal models infected with different microorganisms to find a chemical that would show anti-microbial effects while sparing the animals. Ehrlich described

this differential affinity as high parasitotropism with low organostropism, i.e. a more profound preference for the microbe than for the host. Compound number 606, arsphenamin, was discovered in this test series with these desired effects and later used for the treatment of syphilis under the name “Salvarsan” (Sepkowitz, 2011).

This medical breakthrough, although not immediately appreciated by his peers, marked the birth of Paul Ehrlich’s “magic bullet” concept, the idea of drugs that act specifically on a defined cell type or microorganism without adverse effects on otherwise healthy tissue (Strebhardt & Ullrich, 2008). Ehrlich described the application of chemicals to treat the pathogenic cause of a disease as “chemotherapy”, opposing the traditional field of “pharmacology” which describes the therapy of symptoms alone. Along this new definition, he also formulated the “chemotherapeutic index” as the ratio of the maximum tolerated dose to its minimum effective dose, depicting the affinity of a molecule for its intended target versus its activity on other targets, thereby laying the foundation for the development of early cancer chemotherapy (Dale, 1924).

1.1.2 The dawn of anticancer chemotherapy

Although Paul Ehrlich tried to employ his groundbreaking insights from antimicrobial research for the treatment of cancer and his discoveries and postulations influenced many scientists at the time to do the same, a major limitation stood in the way of developing the first anticancer chemotherapeutics. The lack of appropriate model systems made it impossible to filter the vast number of molecules for effectiveness in cancer and the first cancer cell line was not available before George Gey isolated HeLa cells in 1951 (Scherer *et al*, 1953). After Ehrlich had published the Salvarsan study results, the search for standardized tumor models started to move rapidly and George Clowes developed the first tumor transplant model in rodents at Roswell Park Memorial Institute (Krahl, 1959). For the first time, it was possible to systematically test a larger number of chemicals for their antitumor activity. Further models followed and in 1935 Murray Shear at the US Public Health Service set up the first comprehensive cancer drug screening system using 3,000 compounds in a murine sarcoma model using a highly collaborative national and international approach (DeVita & Chu, 2008).

The modern era of chemotherapy, however, started with the devastating events of both world wars. The disastrous damage caused by chemical warfare on the battlefields of World War I and a bombardment of the SS *John Harvey* at Bari Harbor in Italy during World War II, leading to the spill of liquid sulfur mustard that originated from the ship’s mustard gas bombs, resulted in a close investigation of the effects of warfare gases on the human body (Pratt *et*

al, 1994). Autopsies that were carried out in the aftermath of the Bari incident showed a dramatic depletion of the victims' bone marrow and lymph nodes. In the belief that nitrogen mustard could specifically target myeloid and lymphoid cells, Yale pharmacologists Louis Goodman and Alfred Gilman injected the agent into mice harboring transplanted lymphoid tumors, a cancer that is characterized by the uncontrolled growth of lymphocytes. When they detected a dramatic regression of the tumor, Goodman and Gilman convinced Gustaf Lindskog, a thoracic surgeon, to administer mustine, a prototype nitrogen mustard compound, to a patient with Non-Hodgkin lymphoma in 1943. The short-lived but significant anti-tumor effect in this and other lymphoma patients paved the way for further studies of related molecules with less host toxicity (Einhorn, 1985; Pratt *et al*, 1994; Smith, 2017). Drug development efforts regarding the refinement of these so-called alkylating agents led to a variety of new compounds, many of which are still in clinical use today. Alkylating agents form covalent bonds with the nucleophilic centers of macromolecules, e.g. DNA bases, via their alkyl groups. These bonds can either result in inter- or intra-strand crosslinks, as is the case for bifunctional alkylating agents that form bonds with two DNA bases or result in a linkage of the compound to one DNA base only when agents with a single alkyl group are applied. Consequently, DNA replication is impaired and cells die due their inability to replicate (Siddik, 2005). The macromolecular targets of alkylating agents, however, do not differ between cells. There is no preference of these drugs for cancer cells over normal cells which means that the beneficial cytotoxic effects seen in cancer treatment are mostly due to the intense requirement of rapidly dividing cancer cells for a functional DNA replication machinery, but these drugs do not spare healthy cells dividing at a similar speed.

Another wave of new anticancer drugs can be traced back to nutritional research during the late 1920's. As a visiting researcher in India, Lucy Wills investigated new treatment strategies regarding macrocytic anemia in pregnant textile workers, a condition that is characterized by an increased volume of erythrocytes in association with a global suppression of normal red blood cells. Since the prevalence of macrocytic anemia was highest in poorer populations with diets lacking fruit, vegetables, and protein, Wills experimented with different nutrient supplements. She found that the addition of yeast or yeast extract to diets lacking vitamin B12 were important for bone marrow function and cured the macrocytic anemia in these patients. The curative substance contained in yeast was identified as folate and later synthesized in its crystalline form as folic acid (Hoffbrand & Weir, 2001).

Since patients with acute leukemias oftentimes present with serum folate deficiency, the first physicians begun to experimentally apply folic acid, hoping that a sufficient supply of folate would cure the disease, as it did with macrocytic anemia. Among these early adopters was Sidney Farber, at the time Professor of Pathology at Harvard Medical School and pediatric pathologist at the Children's Hospital in Boston. When administering folic acid to 90 children

diagnosed with different malignancies, he was struck by the observation that the tumors showed an accelerated growth with very late regression, attributed to a folate deficiency in the densely packed tumor. Farber came to the conclusion that the folate deprivation within these tumors and the resulting growth inhibition could be achieved by synthesizing a molecule that has the opposite effect of folate, an antimetabolite or folate antagonist. Yellapragada Subbarow, a Professor of Biochemistry at Harvard Medical School and also the first person to synthesize folic acid together with Brian Hutchins and associates was able to produce sufficient amounts of 4-aminopteroyl glutamic acid, also known as aminopterin. Farber applied the antagonist to 16 children diagnosed with acute undifferentiated leukemia, most of which were at a terminal stage. Temporary remission was observed in 10 children including clinical, hematological and pathological improvements. The finding was published in the New England Journal of Medicine in 1948 and was based on the molecule originating from the first rational drug design of an antimetabolite in history. The authors underlined that the remission was of temporary nature and that aminopterin was toxic and could cause even more problematic side effects. Although the results provoked a lot of criticism and resistance, the use of metabolite antagonists became a success story and the aminopterin derivative methotrexate is the most frequently used antimetabolite in cancer treatment nowadays (Farber *et al*, 1948; Miller, 2006; Ribatti, 2012).

The molecular function of folate, however, was not uncovered before the 1950's and 1960's. Folate is a precursor of tetrahydrofolate (THF), which represents a central hub in the one-carbon metabolism. Besides supplying methyl, methylene, and formyl groups in biochemical reactions, THF plays an important role in methylation of homocysteine to methionine and is indispensable for DNA replication through its participation in the purine and deoxythymidine monophosphate synthesis pathway (Newman & Maddocks, 2017). In the case of macrocytic anemia, folate deficiency through malnutrition can result in impaired DNA synthesis due to a shortage of the folate-derived DNA bases in the fast-dividing hematopoietic precursor cells. While RNA and protein synthesis are less affected and work at a higher speed than the synthesis of DNA, S-phase is prolonged and this maturation arrest causes a major increase in cytoplasmic constituents, defects in cell division, and unbalanced cell growth (Green & Datta Mitra, 2017). Since folate is required in cell types that rely upon a fast rate of DNA synthesis, folate supplementation can enhance cell division rates in cells that are otherwise facing a metabolic bottleneck, as was observed by Sidney Farber and his colleagues. Antimetabolites like methotrexate block DNA synthesis and have cytotoxic effects on rapidly dividing cells such as cancer cells, but also on otherwise normal cells such as benign types of the hematopoietic system, hair follicle cells or epithelial cells of the gastrointestinal tract, contributing to the well-known side-effects of anticancer chemotherapy, like pancytopenia, alopecia, or nausea, among many others (Visentin *et al*, 2012).

The past 70 years of chemotherapy research have yielded numerous drugs targeting additional cellular processes that present a vulnerability of cancer cells. Besides the already discussed inhibition of DNA synthesis through antimetabolites such as methotrexate and the impairment of DNA replication using alkylating agents, the class of mitotic inhibitors like *vinca* alkaloids aims at impairing tubulin function, thereby interfering with mitotic spindle formation and distribution of sister chromatids into newly formed cells, which results in cell death (Portugal *et al*, 2010). Certain antibiotics like anthracyclines target the topoisomerase class of enzymes. These proteins contribute to the topological changes of DNA during cell cycle via controlled double-strand breaks of supercoiled DNA and subsequent re-ligation of the strands. The inhibitors act either through topoisomerase-associated promotion of double-strand breaks or by inhibiting the DNA ligation function of these proteins, giving rise to an accumulation of double-strand breaks and cell death (Pommier, 2013). Combinational treatment approaches with different chemotherapeutics as spearheaded by Emil Frei and Emil Freireich (Devita *et al*, 1970; Levitt *et al*, 1972) and chemotherapy application in an adjuvant or neoadjuvant fashion, i.e. before or after surgical removal of the tumor mass (Bonadonna *et al*, 1976; Moertel *et al*, 1990), as well as progress in medicinal chemistry which allowed the formulation of compounds into drug-like molecules (Chabner & Roberts, 2005), and the advent of cell line panels like the NCI-60 that were used for diversified screening strategies (Shoemaker, 2006), all contributed to the rise of chemotherapeutics and the inherent success stories of fighting, and often curing, diseases that used to be synonymous with a death sentence.

The commonality between these drugs is that they target cellular processes not exclusive to cancer but shared by healthy cells in the body as well. Aggressively moving in on rapidly dividing cells by impairing functions that are essential for the high speed of proliferation gives rise to numerous adverse complications which, although potentially life-saving, turn chemotherapeutic drugs into a double-edged sword. Side-effects range from anemia (Groopman & Itri, 1999), myelosuppression and immunosuppression (Rasmussen & Arvin, 1982; Kurtin, 2012), neutropenic enterocolitis and gastrointestinal distress (Mitchell, 2006; Nesher & Rolston, 2013) as well as nausea, vomiting, and hair loss (Trüeb, 2010; Janelsins *et al*, 2013) to infertility due to gonadotoxicity and therapy-related ovarian damage (Schrader *et al*, 2001; Imai & Furui, 2007). Pregnancies in the first trimester are usually terminated during chemotherapy due to the teratogenic effects of many of these drugs (Koren *et al*, 2013). Life-threatening adverse events include organ damage such as hepatotoxicity, cardiotoxicity, or nephrotoxicity (Vogelzang, 1991; Floyd *et al*, 2006; Colombo *et al*, 2013), but also secondary neoplasms are frequently detected after exposure to chemotherapeutic agents (Krishnan & Morgan, 2007). Often, these adverse events force physicians to limit drug treatment to doses that are not efficiently killing cancer cells anymore, worsening risk-benefit-ratio for patients. Moreover, chemotherapy frequently is not curative and multiple lines of treatment need to be

applied. Resistance mechanisms come into place, either initially present in a clonal population within the tumor mass or acquired through the mutagenic effects of many drugs, making treatment with the standard repertoire of anticancer chemotherapeutics ineffective (Housman *et al*, 2014).

The lack of cell specificity as a cause of these dramatic side-effects has been a major hurdle in cancer treatment since the early days of chemotherapy. Interpretations of Paul Ehrlich's magic bullet concept have called for a new type of anticancer drug which would target a single protein that is exclusive to cancer cells. The identification of tumor suppressors and oncogenes in the 1970's and 1980's as well as the deconvolution of associated signaling pathways sparked enthusiasm among the scientific community for new targets in cancer therapy and novel compounds with less toxicity for patients. At the same time, the "war on cancer", as proclaimed by US president Richard Nixon and manifested in the National Cancer Act of 1971 with the goal to eradicate cancer as a major cause of death would propel efforts in developing more efficient treatments. The era of targeted therapy was on the rise and should change the field of oncology dramatically.

1.1.3 The discovery of oncogenes

The origin of cancer probably dates back to a time even before the human race started to populate the planet, as indicated by paleopathological studies (Rothschild *et al*, 1999). The first written description of human cancer originated in 3,000 BC and was recorded in what became known as the Edwin Smith Papyrus. The ancient Egyptians generated a systematic review of trauma injuries and diseases, among which they found a tumor mass in the breast to be life-threatening condition with no available treatment (Hajdu, 2011). The molecular foundation of the disease remained concealed during the following millennia and even at the dawn of chemotherapy there was still a dramatic lack of knowledge about the pathophysiology of cancer.

When Peyton Rous discovered that a tumor cell free isolate from transmissible chicken sarcomas could induce further tumor growth in secondary chicken in 1911, he challenged the axiom that cancer is an endogenous disease (Rous, 1911). The Rous-sarcoma virus (RSV), named after Rous, was later shown to be responsible for the observed malignant transformation of cells and that one of its genes, v-*src*, was the molecular driver and preserver of the transformed phenotype (Martin, 2001). With *src*, the first oncogene was confirmed in 1970 and its nucleotide sequence was published ten years later (Czernilofsky *et al*, 1980). One of the most influential findings in oncology research however was not only the fact that a

single viral gene can cause neoplastic transformation but that this gene, v-*src*, had a related sequence in the original chicken cells, termed c-*src* for "cellular" *src*, which becomes incorporated into the viral genome through recombination during the virus life-cycle. Moreover, similar sequences could be identified in different avian species and contributed to the oncogene theory which illustrates the wide-spread presence of proto-oncogenes in healthy cells, i.e. critical genes responsible for cellular functions such as cell growth and cell cycle, that are transformed into oncogenes by mutation or amplification, thereby leading to uncontrolled growth as well as other hallmarks of cancer (Stehelin *et al*, 1976; Adamson, 1987; Hanahan & Weinberg, 2000, 2011; Bister, 2015).

A variety of human proto-oncogenes has been found at the same time. Some of the first somatic alterations described in the context of cancer development were the three commonly found chromosomal rearrangements in Burkitt's lymphoma. Each of these alterations juxtaposes an enhancer element next to the gene encoding the transcription factor MYC, resulting in constitutive activation of the oncogene (Croce *et al*, 1983). In the same year, transfection experiments of DNA from human cancer cells into mouse fibroblast was shown to induce certain characteristics of malignant cells. The gene responsible for this phenotype was identified as the *RAS* gene. A guanine nucleotide-binding protein, it is central to a multitude of signaling pathways governing cellular proliferation and differentiation and uses the energy generated by GTP hydrolysis to function as a molecular switch. In tumors, *RAS* mutations lead to the protein's inability to hydrolyze GTP, keeping it in a constitutive active conformation which upregulates proliferative signaling and facilitates oncogenic transformation (McCoy *et al*, 1983; Capon *et al*, 1983).

Generally speaking, proto-oncogenes code for proteins that play wide-spread roles in either cell proliferation, apoptosis, or both. Their activation can be a result of chromosomal rearrangements, as seen in Burkitt's lymphoma above, gene fusions via chromosomal translocation, mutations and amplifications. The latter frequently occurs during cancer progression while the former types of genetic alteration are found both at the initiation and progression stage (Vogelstein & Kinzler, 2004; Croce, 2008).

Oncogene products can be roughly grouped into six categories: Epigenetic factors, transcription factors, apoptosis regulators, growth factors, growth factor receptors, as well as signal transducers (Croce, 2008). *MLL1* gene fusions with more than 80 different partners have been identified in acute lymphocytic and acute myelogenous leukemia. MLL in composition with other proteins forms a stable complex which is implicated in remodeling, acetylation, methylation, and deacetylation of nucleosomes. The pathological MLL fusions deregulate the expression of genes encoding transcription factors, receptor tyrosine kinases and microRNAs by interfering with the epigenetic functions of MLL (Milne *et al*, 2002; Hess, 2004).

Transcription factor translocations are frequently found in lymphoid cancer and various sarcomas. Ewing sarcoma, a tumor of the bone and soft tissue often detected in children and young adults, is molecularly characterized by a gene fusion product involving the transcription factor EWS, whose RNA binding domain is substituted by fusion partners such as FLI, another transcription factor, leading to a massive deregulation of transcription and an elevated cell proliferation rate (Uren & Toretsky, 2005; Johnson *et al*, 2017).

Apoptosis pathways are often deregulated in many tumors and genes encoding for members of these pathways represent proto-oncogenes as well. Catalytic cleavage activates caspases which are essential for controlled apoptosis. This activation can be initiated through two main pathways, the extrinsic, or death receptor, pathway and the intrinsic, or mitochondrial, pathway (Figure 1). As the name suggests, the extrinsic pathway relies on extracellular stimuli which bind to death receptors on the cell surface, such as TRAIL1 (tumor necrosis factor related apoptosis-inducing ligand receptor 1), TRAIL2, FAS, or TNFR1 (tumor necrosis factor receptor 1). Adaptor-proteins such as FADD (FAS-associated death domain protein) link initiator pro-caspases 8 and 10 to the ligand-bound receptors, forming the death inducing signaling complex, or DISC. The inactive pro-caspases become active through autocatalytic cleavage and further cleave and activate the effector caspases 3 and 7, ultimately leading to apoptosis. On the other hand, the intrinsic (mitochondrial) pathway is frequently altered in cancer. It receives its activation signals from a wide array of intracellular stimuli such as ER stress, metabolic stress, viral infections, DNA damage, nutrient deprivation, but also oncogene activation and developmental cues, eventually causing mitochondrial outer membrane permeabilization, or MOMP. As a result, proteins such as cytochrome *c* and SMAC (second mitochondrial-derived activator of caspases) are released from the mitochondrial intermembrane space. Together with APAF1 (apoptotic protease activating factor 1), cytochrome *c* forms the apoptosome complex, a large protein structure that triggers the activation of inactive pro-caspase 9 and subsequently leads to the cleavage of caspases 3 and 7 and subsequently to apoptosis. Since the formation of MOMP is a crucial and seldom reversible event in the initiation of apoptosis, the cell contains multiple regulators, activators and safeguards acting in balance to inhibit uncontrolled mitochondrial membrane permeabilization (Croce, 2008; Portt *et al*, 2011; Ichim & Tait, 2016). These gatekeepers all belong the family of BCL-2 (B-cell lymphoma 2) proteins which can be classified into three groups according to the number of homology domains in their protein sequence: the pro-apoptotic BH3-only (BCL-2 homology domain 3) proteins (e.g. PUMA, BID, BIM) that exert their effects on the pro-apoptotic effector proteins (such as BAX and BAK) upon intracellular pro-apoptotic signal reception, resulting in MOMP formation, and the inhibitory or anti-apoptotic (pro-survival) BCL-2 proteins (e.g. BCL-2, BCL-X_L, MCL-1). BCL-2 proteins can bind to either the pro-apoptotic BH3-only proteins or to pro-apoptotic effector proteins via their

homology domains, thereby inhibiting crosstalk between the two which would otherwise result in MOMP formation and cell death (Youle & Strasser, 2008; Delbridge *et al*, 2016). Upregulation of BCL-2 and other pro-survival members of the BCL-2 family are frequently seen in cancer, for instance chronic lymphocytic leukemia (CLL), where it counteracts the cell's ability to undergo apoptosis. BH3 mimetics such as Venetoclax, harness the role of BCL-2 mediated apoptosis repression by interfering with BCL-2 binding to its pro-apoptotic partners, thereby sensitizing these cells to apoptotic stimuli (Itchaki & Brown, 2016; Inoue-Yamauchi *et al*, 2017).

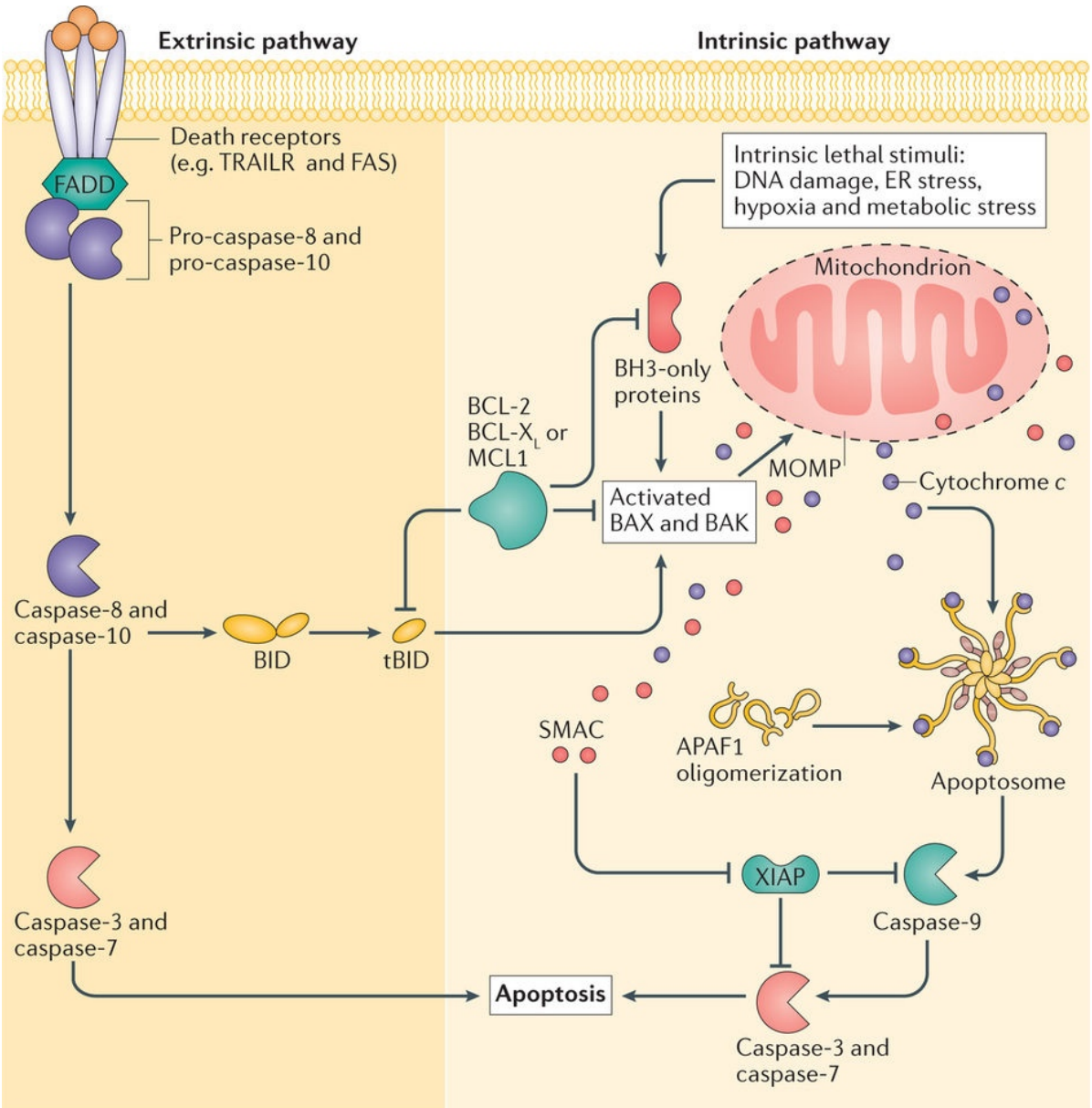

Figure 1 Main pathways of apoptosis. *Apoptotic signaling can either originate from extracellular stimuli (e.g. FasL, TRAIL, TNF) or intracellularly from stress stimuli such as DNA damage, ER stress, hypoxia or metabolic stress. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Ichim & Tait, 2016)*, copyright (2016).

Not only the evasion of apoptosis as seen in oncogenic mutations of BCL-2 family members is a hallmark of cancer but also the self-sufficiency of proliferative signaling (Hanahan & Weinberg, 2000). Proto-oncogenes also encompass the genes coding for growth factors. In contrast to the physiological functions of paracrine growth signaling, such as tissue development during embryogenesis and wound healing, autocrine release of growth factors can contribute to tumorigenic transformation. Many growth factors such as the epidermal growth factor (EGF) family, insulin-like growth factors (IGFs), transforming growth factor β (TGF-β), or the vascular endothelial growth factors (VEGFs) are overexpressed in cancer. This deregulation often leads to clonal expansion of malignant cells, endothelial-mesenchymal transition and metastasis, as well as intra- and extravasation and the promotion of angiogenesis to sustain nutrient supply for tumors (Witsch *et al*, 2011).

Growth factor signaling is dependent on the corresponding receptors, which also show frequent alterations in cancer. Although ligand binding is a prerequisite for receptor activation and signal transduction, many tumors encompass deletions in the receptor ligand-binding domain, rendering them constitutively active, thereby deregulating a multitude of signaling pathways downstream. Alternatively, genetic alterations can cause overexpression of receptors and an accumulation on the cell surface, making the cell hypersensitive to otherwise normal concentrations of growth factors (Scaltriti & Baselga, 2006; Tiash & Chowdhury, 2015).

Stimuli from elevated levels of growth factors and cytokines or an overexpression of growth factor receptors converge at the signal transduction network within the cell. In a healthy state, these pathways are transiently active and forward the extracellular triggers towards the cell nucleus and alter gene expression according to the respective requirements. The components of these signaling networks are, however, often subject to oncogenic mutations themselves, rendering them constitutively active in the absence of receptor ligands. One example of the adverse effects of signal protein hyperactivation is the MAPK/ERK pathway, also known as the Ras-Raf-MEK-ERK pathway. Once activated, Ras, a Small GTPase, triggers the protein kinase function of Raf, which in turn phosphorylates MEK. MEK then activates ERK, leading to further activation of several transcription factors and a hyperproliferative state (Zhang & Liu, 2002; Chang *et al*, 2003). Since signal transduction pathways include many direct and indirect actors the chance of acquiring an activating mutation in one of these proto-oncogenes is relatively high. The deregulated pathways can then act in various roles such as cell proliferation, cell survival, cell metabolism, or cell polarity and migration, genomic instability, and differentiation (Sever & Brugge, 2015).

Unlike proto-oncogenes for which one mutation is sufficient to contribute to cancer development, tumor suppressor genes mainly follow a two-hit hypothesis which was originally formulated by Alfred Knudson. Knudson examined 48 cases of retinoblastoma and found

statistical evidence that this tumor type is caused by two mutational events in the same gene rather than one (Knudson, 1971). Subsequent experiments soon found the commonly mutated region to reside on chromosome 13q14 and named the gene *RB* for retinoblastoma. Through interaction with E2F transcription factors, RB family proteins assist in repressing genes otherwise necessary for DNA replication and transition from G1 to S-phase. If both copies of the *RB* gene are missing, cell cycle regulation is deficient which results in tumorigenesis and carriers of a single mutant allele are highly susceptible to develop further types of tumors (Kleinerman *et al*, 2005; Dick & Rubin, 2013).

Although (proto-)oncogenes and tumor suppressor genes are equally important in the contribution to cancer development and progression, targeting tumor suppressors directly has been proven to be a technical challenge. Restoration of gene function could be attempted by either introduction of a wildtype copy via gene transfer approaches or through small molecule inhibitors that result in gene reactivation but both approaches are either impractical due to toxicity and inefficiency or can only be applied to a very limited cohort of patients (Guo *et al*, 2014). Targeting tumor suppressors indirectly, however, has emerged as an elegant way to overcome some of the current limitations in cancer therapy and will be discussed in one of the following chapters.

1.1.4 Oncogene addiction as a basis for targeted therapy

Strenuous at first, the discovery of novel oncogenes has experienced a sharp rise when next generation sequencing methods became available and affordable enough to sequence the genomes of a wide variety of cancer patients (Shendure *et al*, 2017). One of the most striking observations was that the genomic landscape of cancer consists of mutations in oncogenic drivers, such as tumor suppressors and oncogenes, and mutations that do not obviously contribute to cancer initiation. The latter type, often referred to as passenger mutations, are probably acquired through the phases of cancer progression, mainly due to genomic instability inherent to many cancer types. They do not necessarily possess the capabilities to initiate cancer or maintain its progression but potentially increase cellular fitness in a given environment (Greenman *et al*, 2007). The variety of different mutations hitting both protagonists and supporting acts together with the finding that cancer evolves in a multistage process leading to an accumulation of these genetic alterations over time has emphasized the notion that cancer is not a single disease but hundreds of diseases. Moreover, sequencing studies alone are not capable of determining the driving force behind a certain tumor type and

many of the oncogene hits and tumor suppressors need thorough validation before they take the stage at drug development efforts (Torti & Trusolino, 2011).

Certain oncogenes, however, have been shown to be of utmost importance for tumors to develop and progress and their inactivation leads to cell-cycle arrest, differentiation, or induction of apoptosis. The dependence of tumors on these oncogenes was termed “oncogene addiction” by Bernard Weinstein in 2000 and originally described the observation that certain cancers presenting with high cyclin D expression regress their tumorigenic phenotype after RNAi-mediated (RNA-interference) cyclin D knockdown (Weinstein, 2000, 2002). Evidence for the concept of oncogene addiction was found in many cancer models and the oncogenes that are essential for tumor cell proliferation and survival are important targets for drug development studies. In a murine model, for instance, the transgenic overexpression of MYC in hematopoietic stem cells lead to the onset of T-cell and myeloid leukemias, whereas the subsequent repression of this oncogene resulted in differentiation and proliferative arrest (Felsher & Bishop, 1999). Using antisense oligonucleotides against *K-RAS*, the growth phenotype of human pancreatic cancer cells with mutations in the *K-RAS* gene could be reversed while cell lines with wildtype *K-RAS* did not show these effects (Aoki *et al*, 1997). A similar picture was seen when treating breast cancer cells carrying *HER-2* amplification. Oligonucleotides against the gene coding for the EGF receptor slowed cell proliferation but spared proliferation rates in cells with wildtype protein levels of HER-2 (Colomer *et al*, 1994).

Oncogene addiction represented a new target category in cancer therapy and successful drug development initiatives soon started to yield fruit with Imatinib (Gleevec), a novel tyrosine kinase inhibitor, as the first signal transduction inhibitor introduced into the clinical setting for the treatment of a subtype chronic myeloid leukemia (CML). The reciprocal translocation of chromosomes 9 and 22 generates a fusion protein termed BCR-ABL located on what is called the Philadelphia chromosome which constitutes a hallmark of CML (Melo, 1996). This genetic aberration turns the tyrosine kinase ABL constitutively active, resulting in uncontrolled cell division rates. High-throughput screening approaches in combination with medicinal chemistry efforts resulted in the generation of a BCR-ABL tyrosine kinase inhibitor STI571 (Imatinib or Gleevec). Imatinib selectively targets cells carrying the oncogenic fusion protein by binding to the kinase domain of ABL, thereby inhibiting proliferative signal transduction and pushing the transformed cells towards apoptosis induction. While the ABL kinase in wildtype cells is also inhibited, other tyrosine kinase pathways can compensate for the inhibition but Imatinib-sensitive cells are addicted to the constitutive oncogenic signaling (Druker, 2002; Wong & Witte, 2004). In 2002, Imatinib was granted accelerated approval by the FDA as a first-line treatment for newly diagnosed CML and has shown low side-effects and high efficiency in treating patients with BCR-ABL$^+$ CML (Johnson *et al*, 2003). The essentiality of this fusion protein for cancer cell survival has been strengthened by the

identification of resistance mechanism that allow CML cells to become structurally immune to the treatment with Imatinib. Treatment with small molecule inhibitors like Imatinib can constitute a selective pressure which would convey a growth advantage for cancer cells that are unresponsive to the current medication. Point mutations, for instance, can change the conformation of BCR-ABL in a way that Imatinib is unable to bind, thereby circumventing the block in signal transduction. Resistance mechanisms like this have led to the application of second generation tyrosine kinase inhibitors and underline the validity of the concept of oncogene addiction (Milojkovic & Apperley, 2009; Bhamidipati *et al*, 2013).

Various models try to explain the molecular rationale for the effects of targeting oncogene addictions in cancer. The genetic streamlining hypothesis describes the results of a dominant oncogene on intra- and extracellular functional networks. The constant genetic pressure that cells undergo upon transformation results in a high level of genetic drift, i.e. an altered frequency of genetic variants in the cancer genome by chance. The result is a loss of cellular functions that do not contribute to cellular fitness or viability, generating a dependency on genes that are essential for these processes (Kamb, 2003). As a consequence, perturbations of the main survival and growth pathways can have dramatic anti-proliferative effects in cancer cells since compensatory mechanisms present in wildtype cells might not be functional anymore in the malignant clones. In theory, this cancer-inherent feature could have opposing effects when passenger mutations become essential and begin to confer resistance in response to a selective pressure, such as drug treatment and oncogenic signal cascade inhibition (Torti & Trusolino, 2011).

Another model of oncogene addiction termed the “oncogenic shock” hypothesis aims at describing the apoptosis phenotype seen upon inhibition of the dominant oncogene signaling. As has been discussed in the previous chapter, anti-apoptotic and pro-apoptotic proteins exist in a well-regulated balance in normal cells. Upon induction of apoptotic signaling, the pro-apoptotic portion prevails, leading to controlled cell death. However, the oncogenic shock describes the assumption that malignant cells harboring dominant active oncogenes simultaneously sustain a balance of both the pro-apoptotic and pro-survival signals in favor of the latter and that these proteins underlie a differential attenuation rate upon inhibition of the oncogenic product (Figure 2). When the oncoprotein is disrupted by a small molecule inhibitor, e.g. a tyrosine kinase inhibitor, the downstream pro-survival signals decay much faster and the pro-apoptotic signals then, in the absence of counteracting events, drive the cancer cells towards apoptosis. The ability to adapt to oncogenic shocks is a possible scenario in which cancer cells develop resistance mechanisms to treatment with targeted small molecules (Sharma *et al*, 2006; Weinstein & Joe, 2006; Sharma & Settleman, 2007; Weinstein & Joe, 2008; Torti & Trusolino, 2011).

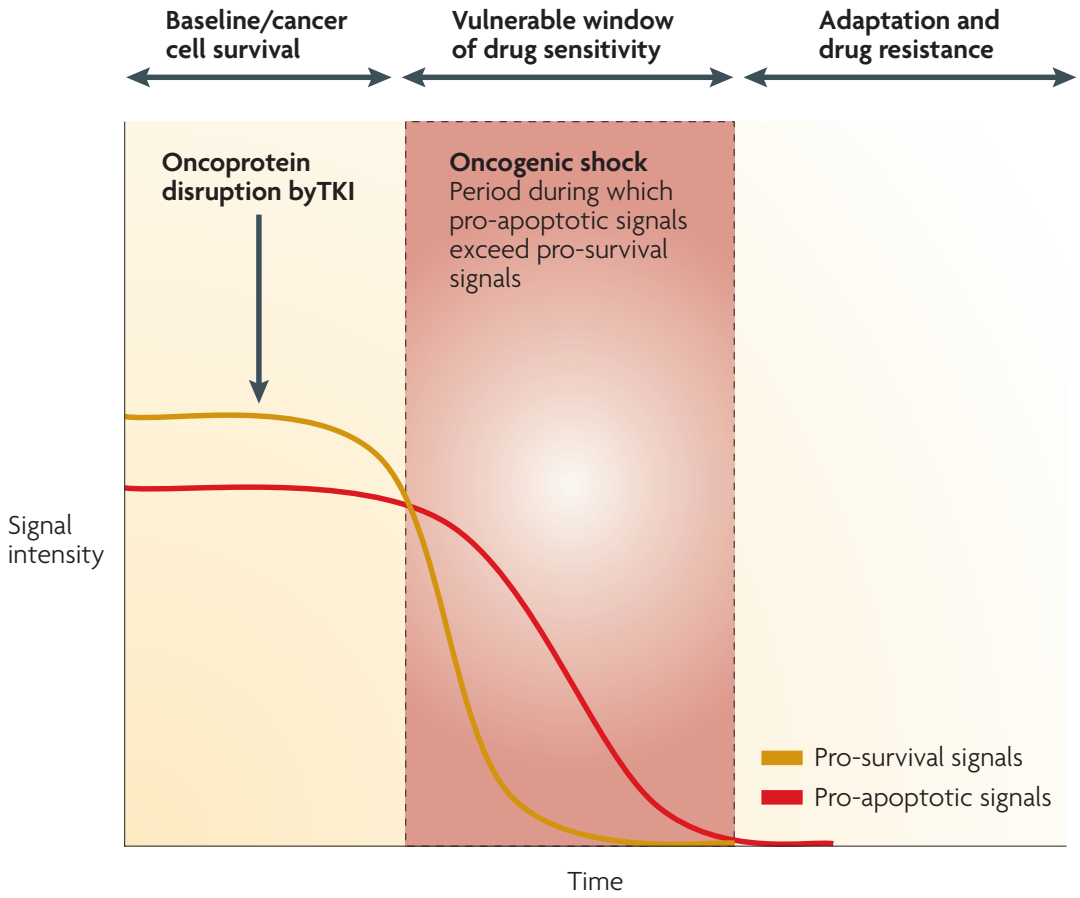

Figure 2 Survival and apoptosis signaling imbalance upon oncoprotein disruption. *Disruption of oncoproteins can lead to an imbalance of pro-survival and pro-apoptosis signals in favor of the latter, a phenomenon called "oncogenic shock". This forces the cancer cells towards apoptosis in the absence of counteracting signals. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Sharma et al, 2007), copyright (2007).*

The concept of oncogene addiction has set the stage for the emergence of highly efficient, cancer cell specific targeted therapies with far less side effects compared to conservative chemotherapy. For the first time, cancer therapy changed from a "one size fits all" approach to a personalized treatment decision. Moreover, many cancers are now manageable, similar to chronic diseases and some can even be cured by exploiting their dependency on oncogenes. It took almost 100 years from Paul Ehrlich's first "magic bullet" Salvarsan to the FDA approval of Imatinib but the speed at which molecular targets are identified and harnessed has massively increased. Nevertheless, the concept of oncogene addiction faces a multitude of limitations and new strategies to tackle cancer at additional Achilles' heels are urgently sought after. Some of the limitations as well as the approaches to overcome these constraints will be presented in the following chapters.

1.2 Identification of novel cancer vulnerabilities – rationale and obstacles

Until recently, genetic testing consisted of cumbersome techniques including the identification, cloning and sequencing of single candidate genes, an endeavor that could only circuitously be applied to large scale examinations of genetic aberrations. Next generation sequencing technologies have enabled the genomic characterization of a wide population of cancer patients and helped to identify a variety of oncogenes and targetable oncogene addiction mechanisms (Garraway & Lander, 2013; Vogelstein *et al*, 2013). Sequencing of thousands of cancer patients and their corresponding normal tissue samples has allowed insight into the tumor-specific frequency of different mutations. It has become evident that the bulk of cancer genes are found at an intermediary to low proportion of 2-20% for a specific tumor with only few oncogenes present at higher frequencies (Lawrence *et al*, 2014). The broad-brush conclusion of this finding is that the "low-hanging fruit"-oncogenes that would drive the majority of a certain cancer patient population have already been identified and that drug development efforts directed towards the mutations with intermediary or low frequency are less tempting from an economic perspective with the current models of drug discovery (Friedman *et al*, 2015). As a result, cancer patients that harbor rather uncommon mutations are disproportionally disadvantaged when it comes to targeted treatment options and many cancers are only treatable by conservative cytotoxic approaches. This development is also reflected in the categories of drugs that were granted approval by the FDA in the past: despite a clear increase in approvals of targeted drugs, almost every third one is targeted against a tyrosine kinase, and this portion does not even include the respective receptors (Sun *et al*, 2017).

Besides the limited number of molecular targets and target classes for which drugs have been successfully approved to date, a common clinical observation is the emergence of drug resistance mechanisms allowing cancer cells to evade therapeutic approaches. Since cancer is not just an accumulation of mutations over time but a slow, multi-step process that is governed by the clonal expansion of the cells the can best cope with extra- and intracellular stresses, it is no surprise that the selective pressure exerted by targeted drugs leads to the outgrowth of resistant clones (Greenman *et al*, 2007). The basis for these resistance mechanisms are wide spread. Drug efflux, for instance, can be upregulated resulting in a lower-than-necessary drug concentration in the tumor cell. The ATP-binding cassette transporter family of proteins (ABC-transporters) has been shown to play a role in the export of targeted drugs and is commonly modulated by tyrosine kinase inhibitors such as Imatinib or Erlotinib (Holohan *et al*, 2013). Alternative alterations like mutations or differential expression can involve the drug target itself. Genomic amplification of the androgen receptor

(AR) is observed in ca. 30% of prostate cancer patients that were treated with androgen deprivation agents or AR inhibitors, resulting in a higher sensitivity to the androgen stimulus and a lower efficiency of inhibitors since more of the receptors need to be interacted with (Palmberg *et al*, 1997). The prime example for this type of resistance mechanism, however, are the T315 residue alterations of the kinase domain of BCR-ABL which render Imatinib unable to bind but simultaneously maintain the oncogenic activity necessary for uncontrolled cell growth (Gorre *et al*, 2001). This led to the successful development and subsequent approval of second- and third-generation inhibitors such as Dasatinib, Bosutinib, and Ponatinib which can target the Imatinib-resistant form of BCR-ABL (Bose *et al*, 2013). Despite of the upstream and on-target resistance mechanisms, there are various downstream and off-target components that can give rise to drug resistance. During the step-wise evolution of cancer accelerated by different selective pressures like drug treatment the emergence of clones harboring activating mutations or amplifications of anti-apoptotic signals like BCL-2 can render the targeted treatment ineffective (Letai, 2008). The importance of a cell's commitment to apoptosis induction can be measured by BH3 profiling which correlates well with the clinical response to cancer therapy in a number of cases (Chonghaile *et al*, 2011). Apart from anti-apoptotic signaling, other pro-survival signals can contribute the resistance phenotype seen in prolonged treatment with targeted drugs. The upregulation of EGFR for instance is often observed as a consequence of drug treatment which shifts the growth signals to another, redundant pathway that can then compensate for the inhibition of the primary target pathway. This phenomenon is termed "oncogenic bypass" and illustrates a common problem in targeting single players in an oncogenic pathway (Holohan *et al*, 2013; Niederst & Engelman, 2013).

As can be seen from these examples, there is a strong need for therapies that tackle new targets. The identification and exploitation of novel drug targets, however, is very burdensome and many obstacles are in the way of developing new, personalized cancer treatments. Soon after the first cohorts of cancer patients had undergone sequencing of their DNA, it became evident that the majority of "driver" mutations, i.e. mutations that were attributed to the tumorigenic phenotype, were in fact genetic alterations of tumor suppressor genes (TSGs) rather than oncogenes (Morris & Chan, 2015). Tumor suppressor proteins represent a difficult target since their loss-of-function phenotype that contributes to cancer development is hardly druggable. Only if the mutated tumor suppressor protein does not undergo degradation immediately and the mutations are highly abundant across the cancer spectrum, small molecule discovery approaches can be reasonable as has been shown in the case of *p53* mutations where a compound, PRIMA-1, was found to restore the wildtype activity of inactivated p53 protein (Lambert *et al*, 2009). This approach might work reasonably well when mutations confer conformational changes that could be reverted by supplementation

with a drug but are far-fetched when the tumor suppressor protein is completely absent, or the restoration of its wildtype conformation will not reactivate its functions. TSGs therefore remain a class of tumor drivers that are difficult to transform into a clinical target directly.

The introduction of target-based drug screening in the 1990's has promised an easy and quick way of identifying new molecules acting against oncoproteins. The information gained from sequencing efforts at this time together with new recombinant technologies gave rise to what was thought to be a highly efficient and rational model of drug discovery with the premise that the target protein was known and thoroughly validated. An increasing level of R&D expenditures did however not match the desired approval rates of new molecular entities, a development which was attributed to regulatory and competitive aspects and is termed "Eroom's law", a broad hint at the reverse development of "Moore's law" of exponential transistor doublings in the tech industry. What is more, the introduction of the target-centric approach coincided with this reduction in productivity, probably due to several reasons (Sams-Dodd, 2005; Scannell *et al*, 2012; Ciallella & Reaume, 2017). The hypothesis of the target-based drug screening was that the interaction of a small molecule with its target protein in a cell-free environment could be indicative of disease modulation in a patient. A thorough understanding of the disease itself, the indubitable identification of a single candidate protein involved in the pathophysiology and a gapless validation of this target are all factors that are required for this approach (Eggert, 2013; Zheng *et al*, 2013). However, the complexity of many malignancies makes it difficult to pinpoint a single oncoprotein that represents a drug target in the majority of the respective cancer patient population due to the genetic heterogeneity found in tumors (Gay *et al*, 2016).

For this reason, industry and academia have partly shifted their efforts towards a less biased model of drug discovery in which the phenotype observed upon perturbation with small molecules or genetic interference is thought to reveal more information about pathobiology and eventually new drug targets (Eggert, 2013; Wagner & Schreiber, 2016). In an analysis published by Swinney and Anthony, the target-based approach succumbed this phenotypic drug screen with 56% of approved first-in-class small molecules discovered in the latter (Swinney & Anthony, 2011). Focusing on oncology drugs, this comparison is in favor of target-based approaches but only due to the high proportion of kinase inhibitors and probably due to the lower and delayed prevalence of phenotypic assays in industry (Eggert, 2013; Moffat *et al*, 2014). This successful application of a hypothesis-driven drug screen when it comes to the process of target and drug candidate identification is based on the circumvention of issues faced when the target is unknown, when the disease biology is not fully understood or when target validation *a priori* is not expedient.

Despite these advantages it is often still troublesome to identify the correct target due to the inherent polypharmacology of most chemical compounds, i.e. the observation that a

drug has multiple targets rather than one selective interaction partner, although certain deconvolution strategies might be applied (Terstappen *et al*, 2007; Zhang *et al*, 2016). Moreover, the validity of phenotype-based screens and experiments are often distorted because of the utilization of cell lines. Although the genetic alterations found in cancer cell lines overall resemble the ones found in tumors, the non-cancerous tumor cells are mostly absent from cell line populations which ignores the tumor microenvironment and its influence on cancers seen in the clinics (Bignell *et al*, 2010; Beroukhim *et al*, 2010; Dittmer & Leyh, 2015). Tumor-derived cancer cell lines are not always similar to the *in vivo* tumor when it comes to the representation of intratumoral heterogeneity in terms of transcription, DNA methylation, and post-translational protein modification (Domcke *et al*, 2013; Goodspeed *et al*, 2016). Furthermore, cell line availability does not equally cover all neoplastic malignancies, that is tumor-derived cell lines from rare cancers tend to be less abundant than cell lines originating from very common tumors. This imbalance leads to a lower statistical significance of rare cell line models and excludes uncommon diseases from thorough drug discovery efforts. Eliminating cell lines as the intermediary by using primary cells from cancer patients for target discovery endeavors interferes with the requirement of large amounts of cells for classical high-throughput screens. Additionally, the problem of statistical significance and a very limited patient population makes this option impractical for high-throughput drug screenings and target identification unless patient cells are artificially immortalized (Hong *et al*, 2016).

The common dilemma of target- and phenotype-based screening for drug discovery is the high cost and large amount of time necessary to transform an original idea to an approved medication, taking at least 10-12 years with an average cost of 1 billion US dollars (Hughes *et al*, 2011; Zheng *et al*, 2013). The basic model of pharmaceutical research and development resembles a funnel in which identification, filtering, and selection processes iterate until the next stage is reached (Figure 3A and B) with target identification and clinical trials representing some of the most time and capital-intensive steps. Due to the exorbitant high attrition rates in this process (Moreno & Pearson, 2013; Hay *et al*, 2014) disease selection for drug development efforts is often tightly linked to the market size for a potential drug. This restriction leads to an ethical issue of not meeting patients' needs for appropriate medication in time, especially, but not exclusively, in the case of rare diseases and less frequent cancer subtypes.

Although the last decades of cancer drug discovery have led to tremendously successful therapies, the decrease in R&D productivity as well as the emergence of drug resistance mechanisms, the obvious disadvantages of traditional target identification and drug development models, the unexhausted potential of tumor suppressor genes as indirect drug targets, and the overall limited number of agents against a multitude of less common

neoplastic malignancies all emphasize the need for novel ways of identifying cancer vulnerabilities and finding drugs to tackle these targets.

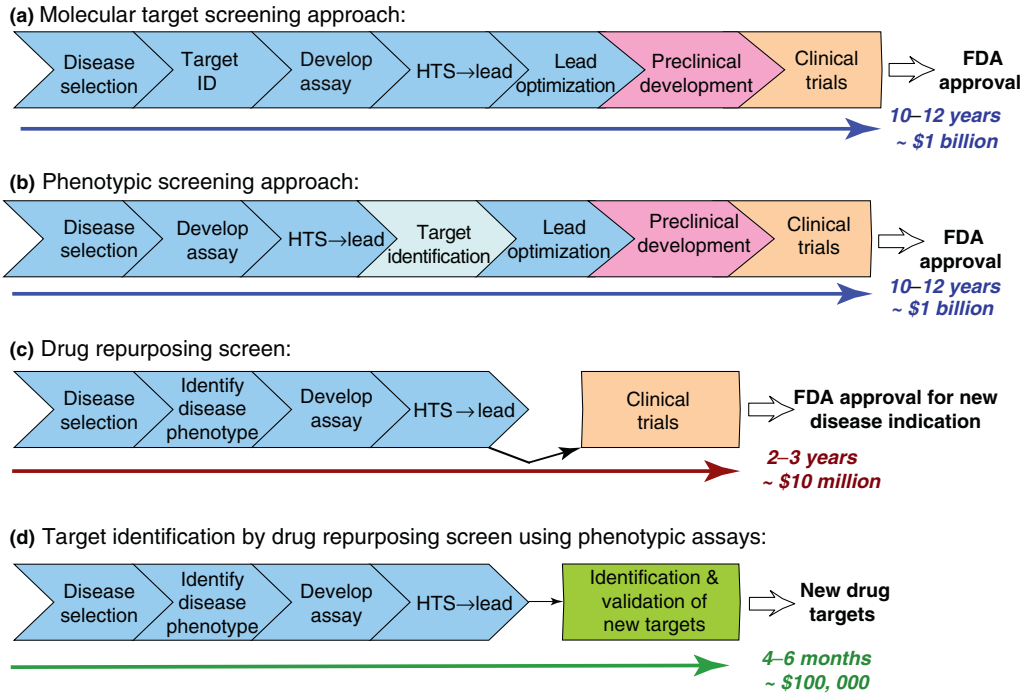

Figure 3 Traditional drug discovery models and drug repurposing screens. *(A) Molecular target screening approaches require a known target. Cost and time estimates average at 1 billion USD in 10-12 years. (B) Phenotypic screening approaches identify the target after the screen but are as costly and time-intensive as target-based screens. (C) Drug repurposing screens have the potential to decrease cost and time investments since preclinical studies have already been performed for the original approval process. (D) Drug repurposing screens can also be applied for target identification in case the mechanism of action of the respective drug is known. Reprinted with permission from Elsevier: Drug Discovery Today* (Zheng *et al*, 2013)*, copyright (2013).*

1.3 Overcoming the obstacles of cancer drug discovery

1.3.1 Drug repurposing in personalized ex vivo drug sensitivity testing

The combination of molecules with a known mechanism of action and primary cells from human tissue biopsies instead of tumor-derived cell lines to discover novel vulnerabilities in cancers has the ability to overcome many of the impediments discussed in the previous section. The use of chemogenomic drug libraries, i.e. a set of well annotated target family-directed molecules, in target- and phenotype-based assays has accelerated the target and drug discovery efforts in the past years. Hits originating from this type of screen suggest that the pharmacological targets and/or the pathways disturbed by the molecule might play a role

in the observed phenotype, based on the notion that similar ligands show high affinity to similar receptors (Caron *et al*, 2001; Bredel & Jacoby, 2004; Klabunde, 2007). Such an approach is, for example, highly informative when a kinase of interest is screened against a library of well characterized kinase inhibitors and can serve as a starting point for further medicinal chemistry endeavors to make a hit molecule more selective. In contrast to CRISPR-mediated gene knockout or RNA interference screens which have the ability to reveal an important target candidate as well, the chemogenomic library sets can be quickly used to investigate alterations that are not readily modelled by genetic perturbation, such as modulations of protein-protein interactions for instance (Jones & Bunnage, 2017). If the screening set consists of drugs that are approved for clinical use, this means that the drug targets and the pathological pathways they are involved in are mostly known and that the drugs can be repurposed, i.e. the field of use of an approved small molecule can be changed to the treatment of another malignancy. Moreover, this approach allows to cover a multitude of different target families with a variety of molecules that have been validated in clinical trials already and are effective in their respective group of malignancies (O'Connor & Roth, 2005). Especially for the pharmaceutical industry, selective small molecule drug libraries using approved compounds have the advantage of massively reducing the time intensive and costly path of drug development and mitigate the attrition rates since the clinically important trials have already been conducted and target identification and validation is less laborious as compared to traditional approaches (Figure 3C and D) (Zheng *et al*, 2013).

The power of chemogenomic drug library screens can even be increased when replacing tumor-derived cell lines with primary cancer cells from patient biopsies, thereby turning this system which is used as a foundation for subsequent drug development into a platform for identifying personalized drug sensitivities and therapy options. Primary cell lines have the tremendous advantage of a close physiological resemblance of the conditions encountered within the tumor mass and even if a cell line originating from a malignancy of interest is not available, functional testing can still be performed. The proximity to *in vivo* conditions also takes into account the genetic inter- and intratumoral heterogeneity of individual patients and integrates the effects of the patient's previous treatments into the phenotype observed in response to the compounds. This is particularly important when it comes to drug resistance since many of these mechanisms are uncovered and promoted by the clonal selection of resistant cells through previous administration of antineoplastic agents (Pan *et al*, 2009; Friedman *et al*, 2015; Shin *et al*, 2017).

Rare malignancies constitute a particularly difficult field of drug discovery and treatment. The low number of patients presenting with a less frequent cancer subtype also affects the extent of studies performed through limited funding for research and target identification as well as a nominal economic market size. This, in turn, leaves these patients

with few opportunities once the last line of therapy fails, if it exists at all. *Ex vivo* drug sensitivity testing with clinically approved drugs on primary patient cells have the potential to elucidate the mechanisms behind a certain infrequent neoplasm and provide a fully validated, ready to use and approved treatment option to patients and physicians on the basis of compassionate or off-label use. Since phenotypic-screenings do not require *a priori* knowledge of a potential target and most of the clinically approved compounds have a known and validated target protein (or multiple thereof), this method reaches two goals at once. First, a new, potentially essential pathophysiological mechanism can be unraveled using an unbiased target identification approach which can disclose valuable information about a disease and spark interest for further research and development efforts. And second, the individual patient profits from the time-saving, efficient, and convenient identification of an already approved drug that could improve her condition dramatically and serve the physician in charge as a personalized therapy option tailored to the genetic and epigenetic background of the patient (Sokolowski *et al*, 2014; Litterman *et al*, 2014; Swinney & Xia, 2014). The feasibility of this approach has been shown multiple times in the past, mainly with cancer entities showing a high prevalence, such as acute leukemias, multiple myeloma, or breast cancer (Kangaspeska *et al*, 2016; Majumder *et al*, 2017; Frismantas *et al*, 2017). Interestingly, the population size used in these studies was big enough to classify patients into categories based on their drug-sensitivity profiles. Correlating this information with pathological, genetic, and transcriptomic analysis of the patient samples allowed to draw conclusions about the molecular basis for drug sensitivity and resistance as well as guidance for physicians to choose the right therapy for an individual patient.

The composition of a chemogenomic library is of utmost importance for *ex vivo* drug sensitivity testing. Various strategies exist to adapt the screening set for the limited amounts of patient material available, trying to optimize parameters like compound number, concentrations and concentration ranges tested, target class coverage, plate design, positive and negative controls or incubation time. Besides the commercially available chemogenomic sets, which range from few hundred kinase inhibitors to thousands of biologically and chemically diverse molecules (Jones & Bunnage, 2017), more compact libraries with less requirements for cell material are better suited for testing patient samples. These can, for instance, consist of a set of clinically approved and/or experimental drugs for a certain disease area or subarea which makes them highly relevant for subsequent clinical application. Concentrations ranges can either span standard screening dilutions, e.g. three-fold dilutions starting from 10 µM, or can be adapted to differential response in patient material. Another road was taken by Licciardello, Boidol, and colleagues who designed a drug library that comprises a representative set of all chemical structures and targets of the entirety of FDA-

approved compounds, adding up to 308 molecules that can be screened at human plasma concentration alone or in combination (Licciardello *et al*, 2017).

As shown by these examples, drug repurposing through *ex vivo* chemosensitivity testing of approved drug libraries can circumvent many disadvantages of traditional screening approaches. It can help to identify new cancer vulnerabilities tackled by already approved drugs and elucidate the essentiality of certain proteins and their pathways in cancer. Moreover, it can assist clinical decision-making and bypass the often laborious and time-intensive path of drug discovery for individual cases, thereby allowing patients to benefit from the available repertoire of therapeutic possibilities.

1.3.2 Exploiting non-oncogene addiction to tackle tumor suppressor mutations

Sequencing efforts have provided a multitude of potential drug targets that are putative cancer drivers. Still, tumor suppressor genes that represent an even bigger target space than oncogenes often present with a loss-of-function phenotype that can hardly be restored in patients (Kaelin, 2005). An alternative approach of finding new targets and therapies is the identification of differential vulnerabilities between wildtype cells and cells that harbor mutations in a tumor suppressor gene, similar to the concept of oncogene addiction. Genetic and epigenetic alterations during cancer development and progression change the molecular "wiring" of cancer cells, thereby making them distinct from their healthy counterparts. This rewiring can lead to the exposure of new Achilles' heels that cancer cells rely on, or in other words are addicted to. Such vulnerabilities are often non-oncogenic in nature, meaning the cancer cells become dependent on an otherwise normal cellular function, a phenomenon that was termed "non-oncogene addiction" (Weinstein, 2002; Luo *et al*, 2009; Nijman, 2011; Nagel *et al*, 2016).

In wildtype cells, the perturbation of a normal, non-essential, cellular protein or pathway can be compensated for by switching to genetically or functionally redundant pathways. However, tumor cells that are molecularly rewired, be it due to the activation of a proto-oncogene or the loss of a tumor suppressor gene, are addicted to this non-oncogenic pathway and can therefore not revert to backup systems. This, in turn, leads to specific growth impairment and cell death of cancer cells but not healthy cells, the result of a phenomenon termed "synthetic lethality" (SL). A synthetic lethal interaction partner that appears in tandem with an oncogenic driver mutation represents an exquisite drug target since only cells carrying the cancer-specific genetic alteration would be sensitive to a small molecule inhibitor.

Harnessing this interaction could result in a superior therapeutic window with less adverse effects (O'Neil *et al*, 2017).

The concept of synthetic lethality was first proposed by Calvin Bridges in 1922 and the term was later coined by Theodosius Dobzhansky in response to breeding experiments with Drosophila flies (Bridges, 1922; Dobzhansky, 1946). It soon became clear that synthetic lethal interactions have the ability to elucidate the network relationships between a multitude of proteins in the cell. Whole-genome screens for synthetic lethality in yeast have immensely contributed to the characterization of protein networks and the annotation of gene functions (Dixon *et al*, 2009). While many of the initial SL screens were performed with the help of large-scale small molecule libraries to identify gene-drug interactions in model systems, the emergence of RNA interference has revolutionized this approach and allowed a systematic mapping of synthetic lethal gene-gene interactions in human (Elbashir *et al*, 2001).

RNA interference describes a biological process in which a double-stranded RNA molecule represses the translation of a gene of interest through binding to and subsequent degradation of its respective mRNA. The introduction of a double stranded RNA homologous to the gene sequence of interest, for instance by viral transduction of a plasmid carrying a short hairpin RNA (shRNA) construct, activates the Dicer protein which recognizes, binds, and cleaves this molecule resulting in short, double-stranded 20nt long small interfering RNA fragments (siRNA). The siRNA fragments are separated into a guide strand and a passenger strand and the guide strand is then incorporated into the RISC complex (RNA-induced silencing complex) where it can bind do complementary mRNA molecules that originate from the transcription of the target gene. This pairing activates the catalytic subunit of RISC, Protein argonaute-2 (AGO2), to cleave the target mRNA molecule, thereby suppressing translation of the messenger RNA (Wilson & Doudna, 2013). Using a pool of double-stranded RNA fragments, for example in the form of viral shRNA vectors or siRNA directly, that cover the entire genome or just a set of functionally interesting candidates (similar to chemogenomic libraries) has allowed a systematic interrogation of synthetic lethalities in human and has revealed valuable information about systems biology and gene-gene interaction networks both in a healthy state and in cancer (Nijman, 2011).

Synthetic lethality to exploit the vulnerabilities of non-oncogene addiction has already been translated into the clinic for the treatment of various forms of cancer. BRCA1 and BRCA2 tumor suppressor genes are frequently mutated in many cancers and predispose to breast, prostate, ovarian, and pancreatic cancers, among others. Both genes are critically involved in double-strand break (DSB) repair via homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) as well as replication fork stability. Loss-of-function mutations in any of the two genes are thought to give rise to the genomic instability phenotype seen in many of these cancers due to impairment of DNA repair and replication stress, consequently

contributing to cancer development (Dhillon *et al*, 2016). Moreover, BRCA-deficient cells show increased sensitivity towards DNA damaging compounds such as platinum-based chemotherapeutics. These agents are successfully used to treat some forms of ovarian cancer. However, the emergence of resistance mechanisms often leads to an unfavorable outcome in the clinics (Fedier *et al*, 2003; Shen *et al*, 2012). In 2005, two groups showed that BRCA-deficient cell lines were severely sensitive to inhibition of poly ADP ribose polymerase 1 (PARP1). PARP1 is involved in single-strand DNA break repair and its inhibition was originally thought to increase the rate of single-strand breaks which rely on homologous recombination repair, a DNA repair pathway that is dysfunctional in BRCA1 and BRCA2 mutated cells (Farmer *et al*, 2005; Bryant *et al*, 2005). An accumulation of single-strand breaks without the necessary reconstitution mechanisms due to BRCA-deficiency leads to chromosomal instability with subsequent cell cycle arrest and apoptosis induction. Other explanations for the mechanism of action have been found for different PARP inhibitors which were observed to "trap" PARP1 at the DNA breakage site through inhibition of PARP1 release, thereby acting similar to topoisomerase II inhibitors and provoking the relevant cytotoxic lesion since BRCA-associated HRR mechanisms would be required to resolve the trap (Pommier *et al*, 2016; Lord & Ashworth, 2017). The dependence of BRCA-deficient cancers on PARP1 illustrates an excellent target exploited by drugs like Olaparib, Rucaparib, or Niraparib since only cancerous cells with loss-of-function mutations of *BRCA1* or *BRCA2* are incapable of HRR while wildtype cells possess functional DNA repair mechanisms of damage induced by PARP inhibition.

Synthetic lethal approaches for the exploitation of non-oncogene addiction in cancer cells has major advantages. The identification of targets that, when inhibited or otherwise perturbed, lead to cell death in a defined mutational background provides a very clear starting point for drug development. Patient populations can be stratified according to biomarkers such as gene mutations or amplifications, as is routinely done in the case of BRCA-deficient breast cancer treatments with PARP inhibitors and adverse events can be kept to a minimum since the drug will only be used for patients that likely respond to the treatment. Additionally, a large enough therapeutic window allows the selective attack of cancer cells with lower drug doses, thereby sparing healthy cells and the overall condition of a patient. Drug development efforts for previously deemed undruggable targets and tumor suppressor genes have a fair chance of experiencing a resurrection since these proteins present excellent opportunities for the identification of synthetic lethal interactions. Synthetic lethal approaches should, however, not replace the current oncogene-focused treatment options but rather complement them in a combinatorial fashion. The probability of a tumor cell clone to become resistant to a combination of treatments that target different proteins in this cell is much lower than the likelihood of resistance to an individual component (Kaelin, 2005; O'Neil *et al*, 2017). Synthetic

lethal interaction screens can also elucidate the role of genes in the cellular network. Genome-scale screens in yeast have shown that synthetic lethal gene combinations are a rich source for creating a comprehensive functional map in which genes that play a role in similar pathways cluster together. The reason for this is that synthetic lethal genes exhibit a functional interaction, implying that they cooperate and affect each other. Moreover, correlation analysis allows to measure the distance of these functional clusters to each other, thereby creating a network of cellular functions based on gene-gene interactions (Costanzo *et al*, 2010; Hillenmeyer *et al*, 2008).

In a similar manner, synthetic lethal screening approaches in human cells can provide valuable information about potential drug targets and their underlying biology. Combining cell lines that carry a genetic alteration of interest, for instance a loss-of-function mutation in a tumor suppressor or an amplification of an undruggable oncogene, with RNAi-mediated knockdown screens can shine a light on potential drug targets and oncogenic and wildtype mechanisms. Just like chemogenomic drug libraries are used to study certain protein sets in detail, one can employ a well-defined library of lentiviral shRNA vectors that target a subgroup of genes belonging to the same functional cohort, e.g. chromatin factors or kinases (Licciardello *et al*, 2015).

Drug repurposing approaches using *ex vivo* patient samples and exploitation of non-oncogene addiction through the identification of synthetic lethal gene-gene interactions are two powerful ways of finding new drug targets, deciphering their biology, and providing patients with a novel opportunity for treatment. Application areas for these complementary approaches are wide-spread since classical targeted therapeutics are limited in scope and limited in number while conservative cytotoxic chemotherapy brings along a myriad of dramatic side effects due to the lack of cancer-cell specificity. The following two examples will illustrate the urgent need for new therapeutics and novel ways to identify targetable proteins.

1.4 Application areas for novel modes of target identification

1.4.1 T-cell prolymphocytic leukemia

T-cell prolymphocytic leukemia (T-PLL) is a rare and very aggressive hematological malignancy characterized by an elevated proliferation of post-thymic prolymphocytes. T-PLL mainly affects elderly patients with a median age of 61 years and a slight ascendancy in males (ratio 4:3). Patients commonly present with hepatosplenomegaly, pleural effusions, skin infiltrations, and generalized lymphadenopathy. Approximately 15% of cases do not show symptoms and although this indolent form of T-PLL can persist for several years, disease progression is certain and can occur rapidly (Dearden, 2006). Diagnosis is commonly based on distinctive clinical, morphological, cytogenetic and immunophenotypic features. Peripheral blood shows a pronounced lymphocytosis with over 100,000 lymphocytes per µl while thrombocytopenia and anemia are observed in half of the patient population. General markers of disease burden, such as serum lactate dehydrogenase and beta 2 microglobulin (β2-MG), are often higher than normal (Dearden, 2012; Zhang & Qiu, 2013). Small to medium-sized prolymphocytes in peripheral blood are a key feature of T-PLL and exhibit a single nucleolus with agranular basophilic cytoplasm and surface protrusions. The bone marrow is characterized by diffuse infiltration of prolymphocytes and reticulin fibrosis, whereas impairment of hematopoiesis varies (Graham *et al*, 2013).

Chromosomal abnormalities are frequent in T-PLL, with the most common alterations involving a reciprocal translocation or inversion of chromosome 14 at the break point regions q11 and q32 observed in 80% of cases. These rearrangements lead to the expression and activation of *TCL-1* (T-cell leukemia/lymphoma 1), a proto-oncogene that is also associated with other mature type T-cell leukemias (Pekarsky *et al*, 2001). During early embryogenesis *TCL-1* is expressed in lymphoid tissues such as spleen, liver, thymus, tonsil, or bone marrow, while its expression is restricted to germ cells and T- and B-lymphocyte precursors in adults (Weng *et al*, 2012). Although its physiological function is unknown, TCL-1 has been shown to interact and co-activate AKT-dependent cell survival and proliferation (Laine *et al*, 2000). Known genetic alterations also include *MTCP-1*, *ATM*, *p-53*, and recent genomic sequencing efforts of T-PLL patient samples have revealed frequent mutations in the JAK-STAT pathway (*IL2RG, JAK1/3, STAT5B*) and in epigenetic modifiers (*EZH2, TET2, BCOR*) (Kiel *et al*, 2014; López *et al*, 2016).

Immunophenotypically, T-PLL is characterized by expression of surface markers such as CD2, CD3, CD5, and CD7, as well as $CD4^+/CD8^-$ (ca. 64%), $CD4^+/CD8^+$ (ca. 21%) or $CD4^-/CD8^+$ (ca. 13%). Strong enrichment of the CD52 antigen on the cell surface can be exploited therapeutically using monoclonal antibodies. T-cell receptor rearrangements are present in all cases and the weak CD3 expression together with a strong expression of CD7 suggests a

differentiation stage between thymic and post-thymic T-cells (Dearden, 2006; Graham *et al*, 2013).

T-PLL is generally very aggressive and has a poor prognosis with a median overall survival of 7 months when treated with conventional therapy. Alkylating agents or combination therapies with CHOP (cyclophosphamide, vincristine, doxorubicin, prednisone) show response rates of approximately 30% but their effects are short-lived in the range of months (Dearden, 2012). The introduction of a CD52-specific antibody, alemtuzumab, has shown a dramatic increase in the number and length of responses. Alemtuzumab, a humanized IgG1 antibody, binds the CD52 antigen which is highly expressed on cells of the male genital tract and mature lymphocytes, monocytes and dendritic cells but not on hematopoietic stem cells. Since T-PLL exhibits a particularly high density of CD52, the antibody reacts preferentially with the malignant cells (Dearden *et al*, 2011). The mechanism of action of alemtuzumab is unknown, *in vitro* studies however show that it can induce complement-mediated and antibody-dependent cellular cytotoxicity and direct apoptosis (Alinari *et al*, 2007). Single agent therapy with alemtuzumab has increased median overall survival up to more than 2 years, although all patients relapse eventually. Autologous and allogenic hematopoietic stem cell transplantation (HSCT) in combination with a CD52-antibody have pushed overall survival rates even further. Allogenic but not autologous HSCT has, in some cases, even shown to be curative but not all patients are eligible and relapsed cases have a very bad prognosis (Dearden, 2012).

Despite the high response rates in the light of recent therapy improvements, the vast majority of T-PLL patients eventually relapse and die. Novel targeted therapies are therefore urgently needed. The small patient population representing less than 2% of all mature lymphocytic leukemias, the lack of cell line models, limited genetic insight into disease development, as well as the dramatic course of disease with strong resistance to conventional chemotherapies make T-PLL a prime application area for drug repurposing using an *ex vivo* chemosensitivity approach to identify new ways of dealing with this devastating disease (Andersson *et al*, 2014, 2017).

1.4.2 Subunit mutations in the SWI/SNF family of chromatin remodelers

Sequencing the genome of tumors has shed light onto many deregulated cellular processes that are exclusively observed in cancer and have elucidated the tight link between chromatin regulation and cancer development and progression. The approval of epigenetic drugs like histone deacetylase inhibitors (e.g. Vorinostat) and DNA methylation inhibitors (e.g. 5-

Azacytidine) for cancer treatment has confirmed the notion that the epigenetic regulation of chromatin is crucial in the genesis of neoplastic malignancies and can be exploited for therapeutic interventions (Jones *et al*, 2016).

The DNA in the nucleus is tightly bound to histone proteins, thereby promoting a condensed but highly ordered chromatin state. Nucleosomes represent the basic unit of chromatin, consisting of 146 bp of DNA tightly wrapped around histone octamers. These histone octamers are in turn composed of two copies of the histone proteins H2A, H2B, H3, and H4 but related proteins can also be incorporated into the genome for regulatory reasons. The “beads-on-a-string” state of chromatin consisting of multiple nucleosomes in a row can be further compacted by histone H1, leading to a less accessible chromatin state, termed the “30 nm fiber”. Despite this compaction, the 30 nm fiber contributes only 0.5% to the actual DNA-to-nucleus compaction, suggesting the existence of various others mechanisms that promote higher order chromatin condensation (Hargreaves & Crabtree, 2011).

The tight compaction of chromatin needs to be dynamically resolved to enable essential cellular processes such as transcription, replication, DNA repair, or recombination. This dynamic chromatin state regulation is achieved by two classes of proteins: ones that modify histone tails covalently or recognize these modifications and protein complexes that use the energy of ATP hydrolysis to remodel nucleosomes. The former type reads, writes, or erases post-translational modifications on the N-terminus of histone proteins, such as the EZH2 histone methyltransferase that can transfer methyl groups onto lysine 27 of histone H3, thereby downregulating the expression of the respective genomic region (Yamagishi & Uchimaru, 2017). On the other hand, ATP-driven chromatin remodelers disrupt the DNA-protein interaction to evict or exchange nucleosomes or slide nucleosomes along the DNA, thereby making chromatin regions more accessible for proteins that need to directly interact with DNA or histones. A tight cooperation of chromatin remodelers with histone modifying enzymes has been shown to be a requirement for many developmental processes and flexible transcriptional regulation (Varga-Weisz, 2001).

Chromatin remodelers are protein complexes characterized by the presence of a core ATPase which uses the energy of ATP hydrolysis to physically remodel chromatin and various non-catalytic subunits at the periphery leading to a high degree of functional specialization. Four distinct families of chromatin remodelers are known in eukaryotes, sharing a high level of conservation from yeast to human, in particular in their ATPase domains: ISWI (imitation switch), CHD (chromodomain-helicase-DNA binding), INO80 (inositol requiring 80), and SWI/SNF (switch/sucrose-non-fermenting). Interestingly, the SWI/SNF family of chromatin remodelers has been highly involved in disease. Recent exome sequencing studies have found genetic aberrations in genes encoding SWI/SNF subunits in over 20% of cancer

patients, grouping them in a similar range as the most frequently mutated gene in human cancers, *p53* (Shain & Pollack, 2013; Tan *et al*, 2015; Pierre & Kadoch, 2017).

The SWI/SNF family of chromatin remodelers was originally discovered in yeast genetic screens with the goal of identifying genes responsible for mating-type switching and sucrose fermentation. Its high degree of evolutionary conservation allowed the identification of homologous proteins in further species such as plants, flies, and mammals. Based on their composition, mammalian SWI/SNF (mSWI/SNF) complexes are further categorized into BAF (BRG1 or BRM-associated factors) and PBAF (Polybromo-associated BAF) complexes. The mSWI/SNF complex is highly polymorphic, consisting of 12-15 subunits encoded by at least 29 genes. BAF contains either one of the two mutually exclusive core ATPases BRM (*SMARCA2*) or BRG1 (*SMARCA4* and highly conserved core subunits such as BAF250A/B (*ARID1A/B*, mutually exclusive), SNF5 (also called INI1, BAF47, encoded by *SMARCB1*), BAF155 (*SMARCC1*), BAF170 (*SMARCC2*). Variant subunits are thought to be important for lineage-specific assembly and include BAF60A/B/C (*SMARCD1/2/3*), BAF57 (*SMARCE1*), BAF53A/B (*ACTL6A/B*), BAF45A (*PHF10*), BAF45B/C/D (*DPF1/2/3*), and ACTB (Hodges *et al*, 2016; Pulice & Kadoch, 2017).

The BAF complex has been shown to bind to 20,000 – 40,000 genomic sites, often spanning regions of 2,000 – 5,000 base pairs, suggesting the presence of multiple complexes at these genomic loci. BAF binding sites are highly correlative with genomic enhancer regions and active promoters as shown by integrative analysis. Additionally, BAF exerts its functions through interaction with a multitude of transcription factors such as STAT3, GATA1, or p63 (Hu *et al*, 2011; Ho *et al*, 2011; Bao *et al*, 2015; Takaku *et al*, 2016), thereby playing important roles in a variety of cellular processes, like cardiac development, pluripotency and self-renewal, or neural differentiation (Ho *et al*, 2009; Yoo *et al*, 2009; Singh & Archer, 2014). For instance, neuronal differentiation is dependent on a change in expression of three BAF subunits leading to a switch and a differential complex composition during the course of cellular differentiation over time (Pulice & Kadoch, 2017).

The notion that BAF complex subunits are putative tumor suppressor genes originally comes from studies in malignant rhabdoid tumors (MRT), a rare childhood cancer of the kidney. Sequencing of MRT patients has revealed the lowest estimation of mutational burden in all cancers with the only recurrent genetic aberrations being bi-allelic inactivating mutations in *SMARCB1* (BAF47 or SNF5) in virtually 100% of cases. Mechanistically, the lack of SNF5 increases the placement of repressive H3K27me3 histone marks through upregulation of PRC2 subunit EZH2, skewing the normal BAF-Polycomb antagonism towards a more repressive state, epigenetic silencing of tumor suppressor gene $p16^{INK4A}$, and enhanced tumorigenesis *in vivo*, which could be reversed by applying an EZH2 inhibitor (Wilson *et al*, 2010). Similarly, *ARID1A* mutant ovarian clear cell carcinoma cell lines are sensitive to the

inhibition of EZH2, probably due to their antagonistic relationship in regulating PIK3IP1, a putative tumor suppressor that controls the PI3K signaling cascade (He *et al*, 2008; Bitler *et al*, 2015). BAF250A (*ARID1A*) is the most frequently mutated gene of the BAF subunit family in human cancers and sequencing studies in ovarian clear cell carcinoma and endometriosis-associated ovarian carcinomas have identified its mutation rate of almost 50% (Wiegand *et al*, 2010; Jones *et al*, 2010). Earlier findings that the loss of this tumor suppressor confers sensitivity to inhibition of the PI3K-AKT signaling pathway with small molecules also confirm the Polycomb-related synthetic lethality (Samartzis *et al*, 2014). Recently, *ARID1A* mutations were also found to confer vulnerability to inhibition of the DNA damage checkpoint kinase ATR and histone deacetylase HDAC6, confirming a non-oncogene addiction while emphasizing the role of the BAF complex in DNA damage and replication stress and opening up new opportunities to tackle these mutations in the clinic (Williamson *et al*, 2016; Bitler *et al*, 2017).

The identification of synthetic lethal interactions of loss-of-function mutations in the BAF complex and epigenetic regulators such as EZH2, DNA damage checkpoint kinases, or histone deacetylases have shed light onto the essential networks that chromatin remodelers are connected to. But not only the system-wide ties of the complex can be exploited for identifying treatment opportunities. Besides epigenetic partners, BAF also presents intra-complex synthetic lethality of mutually exclusive subunits. The reason for this is the observation that the loss of a certain subunit can be compensated by another subunit and that oftentimes the complex does still assemble normally even if a subunit is not expressed anymore. For instance, loss of BAF250A leads to the incorporation of its paralog BAF250B (*ARID1B*) and knockdown of *ARID1B* in this genetic context results in synthetic lethality (Helming *et al*, 2014). *SMARCA4* (BRG1), a gene coding for one of the two mutually exclusive ATPase subunits, is frequently mutated in a wide variety of cancers such as tumors of the breast, stomach, lung, bladder, colon, ovaries, and many more, conferring the status of another tumor suppressor gene in the BAF complex. Knockdown of *SMARCA2*, which can normally compensate for the loss of BRG1, leads to cell death similar to the ARID1A/B synthetic lethality (Hoffman *et al*, 2014). Due to the lack of efficient small molecule inhibitors of the SWI/SNF proteins the question remains whether these effects rely on the complete absence of a functional subunit or whether partial inhibition of the catalytic and/or protein-protein-interaction domain would be sufficient to trigger the synthetic lethal phenotype.

The identification of synthetic lethal relationships of SWI/SNF complex subunits with each other and with additional epigenetic modifiers presents a dramatic breakthrough in the biology of chromatin remodeling. Not only gave it rise to various drug development efforts and early clinical trials in the case of mutated *SMARCB1* and *ARID1A* cancers using EZH2 inhibitors but it also shed light on the role and interconnectedness of SWI/SNF protein assemblies in the epigenetic network. Considering the broad functional landscape of

chromatin remodelers and the wide-spread genome occupancy the number of synthetic lethal interactors is, however, still very low and the focus on one single targetable protein for the treatment of patients with SWI/SNF mutations is insufficient regarding the high possibility of resistance mechanisms taking over. Along with drug development efforts for new potential molecules tackling other known synthetic lethal targets, the quest for additional targets in SWI/SNF mutant cancers is of utmost importance. Given the high incidence of these mutations with *ARID1A* and *SMARCA4* aberrations ranking as the two most frequent aberrations in the BAF complex, the identification of further synthetic lethal targets in this genetic context represents a prime application area for harnessing non-oncogene addiction to exploit tumor suppressor mutations.

1.5 Aims

This thesis aims at identifying new cancer vulnerabilities by employing focused chemical and genetic screening approaches. In the first part of this thesis, biopsies from patients with mainly hematologic malignancies were screened using a well-defined set of FDA-approved drugs and compounds in pre-clinical and clinical development to identify small molecules that could reveal information of the molecular foundation of the disease and instantly be used in the treatment of relapsed or refractory T-cell prolymphocytic leukemia on a “compassionate use” basis. Moreover, the mechanistic principles underlying a T-PLL specific sensitivity were subject to elucidation, allowing conclusions about the mode of action of an active drug in this very tumor entity together with potential explanations about possible resistance mechanisms. A further aim was the clinical application of a candidate molecule in patients with T-PLL and the monitoring of their condition and response during treatment. Furthermore, potential combination partners for a drug that shows high effectiveness in T-PLL were screened for synergistic effects.

The second part of this thesis was directed at the identification of new synthetic lethal targets in cancers with subunit mutations in the SWI/SNF family of chromatin remodelers. Using a lentiviral, epigenome-focused RNA interference library in cell lines deficient for either *ARID1A* or *SMARCA4*, potential synthetic lethal interactors were identified and validated in cell lines that were isogenic for the subunits in question. To pinpoint the target specificity a rescue experiment was performed by introducing silent point mutations into the endogenous target site of a short hairpin RNA targeting the gene of interest. In addition, transcriptomic and chromatin accessibility analyses were aimed at elucidating the mechanism behind a potential synthetic lethality. A drug screen was employed to find small molecules that could enhance the phenotype of knocking down a synthetic lethal target.

The results of the research performed in this thesis are thought, on the one hand, to gain insight into the pathophysiology of a type of leukemia that is extremely aggressive and highly resistant to current chemotherapy while providing additional treatment options for patients with this disease and, on the other hand, to lay the foundation for further drug development efforts towards the generation of small molecule inhibitors of a synthetic lethal candidate in SWI/SNF mutated cancers.

2. Results

2.1 First-in-human response of BCL-2 inhibitor Venetoclax in T-cell prolymphocytic leukemia

Bernd Boidol, Christoph Kornauth, Emiel van der Kouwe, Nicole Prutsch, Lukas Kazianka, Sinan Gültekin, Gregor Hoermann, Marius E. Mayerhoefer, Georg Hopfinger, Alexander Hauswirth, Michael Panny, Marie-Bernadette Aretin, Bernadette Hilgarth, Wolfgang R. Sperr, Peter Valent, Ingrid Simonitsch-Klupp, Richard Moriggl, Olaf Merkel, Lukas Kenner, Ulrich Jäger, Stefan Kubicek, and Philipp B. Staber

T-cell prolymphocytic leukemia is a rare and very aggressive T-lymphoid malignancy with fatal outcome. Patients generally respond very poorly to chemotherapy treatment, resulting in a median survival of only 7 months. Despite the introduction of a CD52-specific antibody therapy with alemtuzumab, all patients relapse eventually. To gain insights into the pathophysiology of and find new treatment options for T-PLL, we have established an *ex vivo* chemosensitivity screening system that allows drug response profiling of patient material. Functional drug testing and protein expression profiling of 86 patient biopsies with hematologic malignancies uncovered Venetoclax, a specific BCL-2 inhibitor, to show the best differential response when comparing T-PLL to non-T-PLL samples. BCL-2 expression correlated with Venetoclax response *ex vivo* in all analyzed samples hinting at the on-target effects of the compound. The first in human use of Venetoclax in 2 refractory / relapsed T-PLL patients showed beneficial clinical effects, as evidenced by various clinical parameters and imaging techniques. Following treatment, we could detect elevated expression levels of BCL-2 and BCL-X_L in T-PLL patients, serving as a potential explanation for resistance mechanisms. These results were published in detail in the journal Blood and are reprinted below. Additionally, we have performed a drug combination screen and identified various FDA-approved compounds as synergistically active in combination with Venetoclax *ex vivo*.

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Brief Report

blood

CLINICAL TRIALS AND OBSERVATIONS

First-in-human response of BCL-2 inhibitor venetoclax in T-cell prolymphocytic leukemia

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Key Points

- Strong responses to venetoclax separate T-PLL from other hematologic malignancies in high-throughput drug screening of clinical samples.
- Two relapsed and refractory T-PLL patients demonstrated clinical response on venetoclax treatment.

T-cell prolymphocytic leukemia (T-PLL) is a rare and aggressive T-lymphoid malignancy usually refractory to current treatment strategies and associated with short overall survival. By applying next-generation functional testing of primary patient-derived lymphoma cells using a library of 106 US Food and Drug Administration (FDA)-approved anticancer drugs or compounds currently in clinical development, we set out to identify novel effective treatments for T-PLL patients. We found that the B-cell lymphoma 2 (BCL-2) inhibitor venetoclax (ABT-199) demonstrated the strongest T-PLL–specific response when comparing individual ex vivo drug response in 86 patients with refractory hematologic malignancies. Mechanistically, responses to venetoclax correlated with protein expression of BCL-2 but not with expression of the BCL-2 family members myeloid cell leukemia 1 (MCL-1) and BCL-XL in lymphoma cells. BCL-2 expression was inversely correlated with the expression of MCL-1. Based on the ex vivo responses, venetoclax treatment was commenced in 2 late-stage refractory T-PLL patients resulting in clinical responses. Our findings demonstrate first evidence of single-agent activity of venetoclax both ex vivo and in humans, offering a novel agent in T-PLL. (*Blood.* 2017;130(23):2499-2503)

Introduction

T-cell prolymphocytic leukemia (T-PLL) is an aggressive T-lymphoid malignancy characterized by proliferation of postthymic prolymphocytes.[1,2] Patients typically present with elevated and exponentially rising lymphocyte counts along with splenomegaly, hepatomegaly, lymphadenopathy, and effusions.[1] Responses to alkylating agents or polychemotherapy are poor with a median survival of just 7 months.[2] The use of the monoclonal anti-CD52 antibody alemtuzumab has improved response rates to 75% or even higher when applied in the first-line setting.[3-6] The combination of chemotherapy regimen fludarabine, mitoxantrone, cyclophosphamide (FCM) followed by alemtuzumab increased responses to 90%.[7] However, despite high response rates all patients eventually relapse with a median progression-free survival of <12 months.

Cytogenetic abnormalities involve chromosome 14 and also frequently chromosomes 8, 11, and 17, altering activities of oncogenes and tumor suppressors (*TCL-1*, *MTCP-1*, *ATM*, *TP-53*).[8-10] Next-generation sequencing identified additional mutations affecting the JAK-STAT pathway (*IL2RG*, *JAK1*, *JAK3*, *STAT5B*) and genes encoding epigenetic regulators (*EZH2*, *TET2*, *BCOR*).[11-15] However, these recurrent genetic alterations in T-PLL did not correlate with ex vivo responses to specific drug classes.[16] To overcome the lack of genomically informed therapy, we here applied direct ex vivo chemosensitivity testing to identify effective agents for T-PLL patients.

Study design

Patients and ex vivo drug screen

Samples were obtained and processed after consenting under protocols EKNr1830/2015 and EKNr2008/2015 approved by the local ethics commission. Venetoclax was provided by AbbVie as part of a single-patient preapproval access program. All patient samples (bone marrow aspirates, peripheral blood, or excised lymph node) were prepared freshly and used for drug-screening analysis

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The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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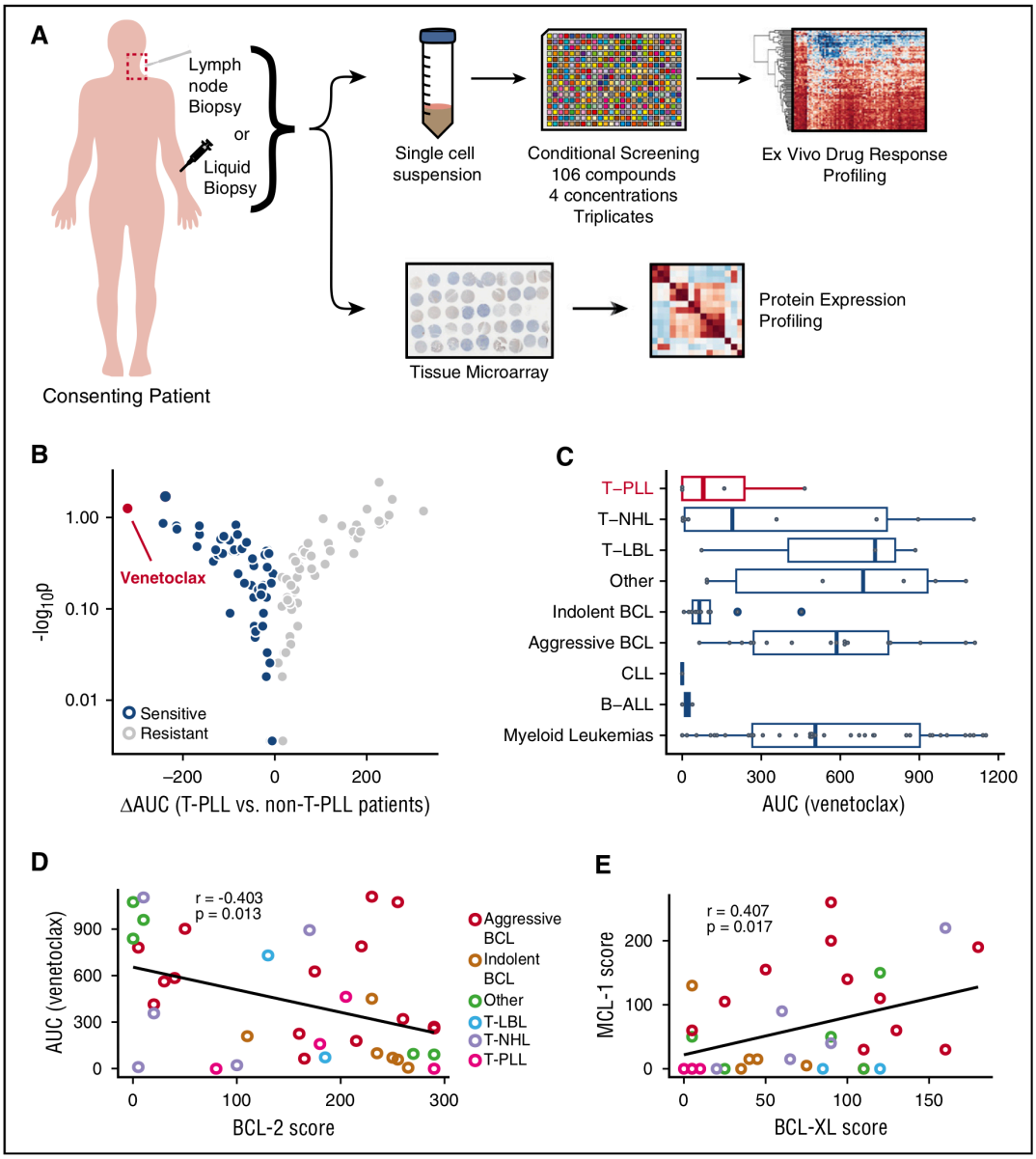

Figure 1. Venetoclax shows strongest differential ex vivo response in T-PLL samples and is correlated with BCL-2 expression. (A) Lymph node and liquid biopsies were taken from consenting patients and used for expression profiling via TMAs and ex vivo drug-response profiling (n = 86). (B) Volcano blot, demonstrating mean differential AUCs (ΔAUCs) plotted for individual compounds comparing ex vivo effects in T-PLL vs non-T-PLL samples (n of compounds = 106). Blue circles indicate compounds hitting more specifically T-PLL than non-T-PLL samples (negative ΔAUCs). Gray circles indicate compounds hitting more specifically non-T-PLL than T-PLL samples (positive ΔAUCs). Venetoclax is highlighted in red as the most specific T-PLL compound identified in the drug screening. (C) AUCs of venetoclax in individual ex vivo samples stratified by indication (n = 86). (D) Comparison of AUCs of venetoclax and corresponding BCL-2 expression score by indication (n = 37). (E) Correlation of MCL-1 and BCL-XL expression scores of individual samples (n = 34). B-ALL, B-cell acute lymphoblastic leukemia; T-LBL, T-cell lymphoblastic lymphoma; T-NHL, T-cell non-Hodgkin lymphoma.

within 3 hours of harvest. Resulting single-cell suspensions (in RPMI 1640 plus 10% fetal calf serum) were seeded at 1×10^5 cells per well in 384-well plates containing compound libraries with each drug in 4 concentrations in triplicate (supplemental Table 1, available on the *Blood* Web site). Drug-concentration range was determined by dose-response experiments in primary cells and cancer cell lines for each compound. Differential cell viability was determined after 72 hours with CellTiter-Glo (Promega) on an EnVision plate reader (PerkinElmer). Dose-response curves and immunoblotting were performed on viably frozen samples. Viability data of these samples after thawing was determined by the CASY Cell Analyzer (OLS) (supplemental Figure 3).

Tissue microarray

Tissue microarrays (TMAs) were generated on a Galileo CK3500 tissue microarrayer (Integrated Systems Engineering) using formalin-fixed paraffin-embedded material (supplemental Table 2).

Data calculation and statistics

Drug responses normalized as percentage of controls were calculated as areas under the curve (AUC) as the sum of all data points for a respective drug in

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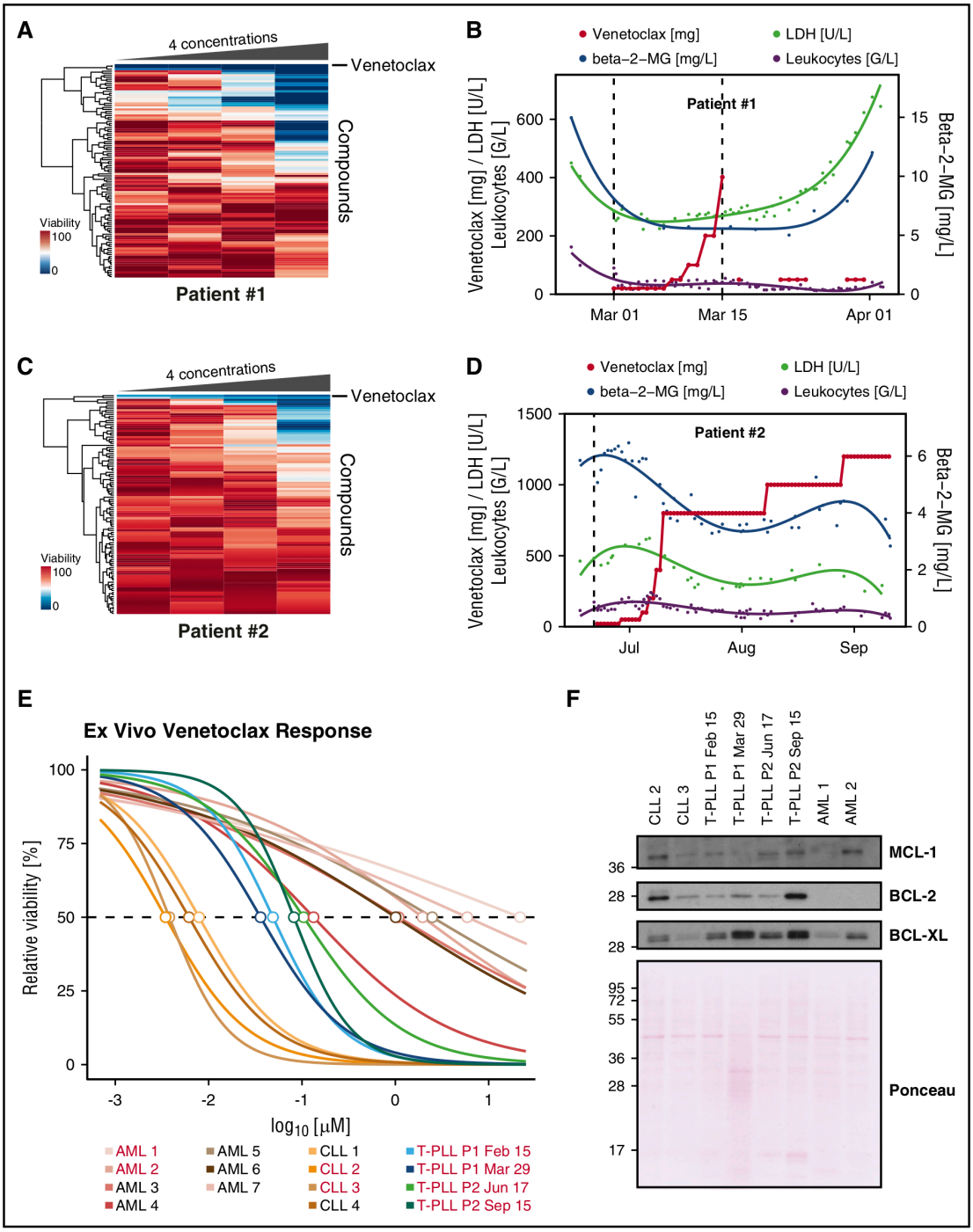

Figure 2. Venetoclax ex vivo response matches clinical response in 2 T-PLL patients. (A) Heatmap of viability measurements for 106 compounds in 4 concentrations in patient 1. The 4 concentrations used for each compound are depicted in supplemental Table 1. Venetoclax clusters on top. (B) Clinical response of T-PLL patient 1. (C) Heatmap of viability measurements for 106 compounds in 4 concentrations in patient 2. Venetoclax clusters on top. (D) Clinical response of T-PLL patient 2. (E) Dose-response curve of venetoclax of patients 1 and 2 before and after venetoclax treatment as well as CLL and AML samples. Concentrations ranging from 0.7 nM to 13 μM in threefold dilutions at 10 concentration points with 8 replicates each. Samples used for western blot in panel F are marked in red. (F) Western blot for BCL-2, BCL-XL, and MCL-1 of samples used in panel E. Total protein stain was performed with Ponceau S. Antibodies used were: BCL-2 (BD Biosciences), MCL-1 (Cell Signaling Technology), BCL-XL (Cell Signaling Technology), horseradish peroxidase (HRP)-conjugated donkey anti-mouse, and HRP-conjugated donkey anti-rabbit (Jackson ImmunoResearch).

individual patient samples.[17] *P* values were calculated using Wilcoxon, Mann-Whitney, and Kruskal-Wallis tests as indicated.

Results and discussion

High-throughput ex vivo drug screening identifies venetoclax sensitivity of primary T-PLL cells

Pioneering studies provided proof of concept that ex vivo chemosensitivities can match clinical response.[16-22] We performed next-generation functional drug profiling on primary cells of 86 patients using 106 compounds (supplemental Table 1). Cell-specific responses were calculated from individual dose-response curves and TMAs were generated for comparative protein-expression profiling (Figure 1A; supplemental Table 2).

To identify specific drug sensitivities for T-PLL, we compared the average ex vivo drug response of T-PLL vs non-T-PLL samples for each drug. The B-cell lymphoma 2 (BCL-2)–selective small-molecule inhibitor venetoclax (ABT-199) demonstrated the strongest differential response for T-PLL (Figure 1B). We also observed strong responses to venetoclax in chronic lymphatic leukemia (CLL) and varying responses in aggressive lymphoma and acute myeloid leukemia (AML) in line with clinical responses and recent reports (Figure 1C).[23-25] Ex vivo responses to venetoclax significantly correlated with BCL-2 protein expression scores (Figure 1D, $P = .013$; negative correlation to AUC, $r = -0.403$), but not with scores for BCL-2 gene family members BCL-XL and myeloid cell leukemia 1 (MCL-1) (supplemental Figure 2A-B). BCL-XL and MCL-1 expression scores demonstrated a significant correlation (Figure 1E, $r = 0.407$; $P = .017$) whereas only MCL-1 appears to be inversely correlated with BCL-2 expression (supplemental Figure 2C-D). T-PLL samples demonstrated strong BCL-2 scores and the most dramatic responses to BCL-2 inhibition by venetoclax (Figure 1D). High expression of BCL-2 and low expression levels of BCL-XL and MCL-1 in T-PLL samples might explain the strong sensitivity toward BCL-2 inhibition (Figure 1D-E; supplemental Figure 2E-F).

Clinical responses of 2 T-PLL patients to treatment with venetoclax

Based on drug and protein expression profiling, venetoclax was administered as an "individual healing attempt" in 2 refractory T-PLL patients. Patient 1, a 58-year-old woman, relapsed within 3 months after 3 treatment lines: (1) FCM, (2) alemtuzumab, and (3) pixantrone, etoposide, bendamustine (PEBen). Fluorescence in situ hybridization revealed deletion 13q and trisomy 12. T-PLL cells presented as clonal prolymphocytes (supplemental Figure 3A). Drug profiling demonstrated strong efficacy of venetoclax (Figure 2A). Before start of venetoclax, the patient presented in poor condition, with splenomegaly (24.7 cm in diameter), lymphocytosis of 161 G/L, and β2-microglobulin (b2MG) and lactate dehydrogenase (LDH) serum levels of 15.1 mg/L and 449 U/L, respectively. Twelve hours upon first venetoclax dosing at 20 mg, lymphocytosis dropped to 28 G/L, b2MG to 7.5 mg/L, LDH to 260 U/L accompanied by laboratory tumor lysis. Overall, venetoclax was tolerated well and resulted in a decrease of splenomegaly from 24.7 cm to 18.3 cm in diameter after 8 days of treatment (supplemental Figure 3B). However, due to severe sepsis, venetoclax had to be stopped after dose ramp-up reached 400 mg after reaching a partial remission as best clinical response (according to criteria described in Dearden[1] and Hopfinger et al[7]). Lymphocyte count remained stable but b2MG and LDH serum levels increased after 10 days and the patient died of fulminant sepsis 15 days after completing venetoclax (Figure 2B).

Patient 2, a 40-year-old man primary refractory after 2 treatment lines ([1] FCM; [2] alemtuzumab) presented with splenomegaly (20 cm in diameter), multiple-stage lymphadenopathy, lymphocytosis (174 G/L), elevated serum levels of LDH (478 U/L), and b2MG (5.1 mg/L). Fluorescence in situ hybridization tested positive for TCL1A translocation, deletion 11q, and trisomy8. Drug profiling indicated response to venetoclax (Figure 2C). The dose ramp-up was well tolerated without tumor lysis. At a daily dose of 800 mg, clinical response could be documented as restitution of splenomegaly, complete regression of lymphadenopathy as evidenced by computed tomography scan, and a significant decrease of all disease-related parameters (Figure 2D; supplemental Figure 3C). After 4 weeks, venetoclax was increased to 1 g for 3 weeks and finally to 1.2 g. However, after 131 days on venetoclax, the patient experienced a relapse-failing salvage treatment with PEBen thereafter. The best clinical response of the patient was a partial remission. High-resolution dose-response curves of venetoclax clearly distinguished CLL, AML, and T-PLL samples ($P = .0024$) (Figure 2E; supplemental Figure 3D). CLL samples responded at already very-low doses (50% inhibitory concentration [IC50], 3.45-7.89 nM), whereas AML samples responded at relatively high concentrations (IC50, 133 nM to 21.7 μM). Responses of T-PLL samples (IC50, 35.98-1040 nM) were stronger compared with AML samples ($P = .0061$) but weaker compared with CLL samples ($P = .0286$). BCL-2 and BCL-XL protein expression was induced upon venetoclax treatment in the 2 patients whereas MCL-1 remained unchanged, thus providing a potential mechanism of venetoclax resistance (Figure 2F). This study demonstrates specific activity of the BCL-2 inhibitor venetoclax in T-PLL. It is noteworthy that a recent ex vivo drug screening also found consistent activities of BCL-2 inhibition in T-PLL, thus confirming these results in an independent cohort.[16] As proof of principle, we report the first-in-human treatment using venetoclax in 2 late-stage T-PLL patients. Responses were striking, however potential mechanisms of resistance might develop through BCL-2 and BCL-XL induction. Therefore, studies testing venetoclax with appropriate combination partners in T-PLL are warranted.

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Authorship

Contribution: B.B. and C.K., performed the experiments; E.v.d.K., N.P., L. Kazianka, S.G., B.H., M.E.M., M.P., O.M., and L. Kenner organized clinical samples and data; G. Hoermann, G. Hopfinger,

M.-B.A., P.V., I.S.-K., R.M., O.M., L. Kenner, and U.J. provided reagents and intellectual contributions; A.H., W.R.S., U.J., and P.B.S. were responsible for patient treatment and ethical guidelines; S.K. and P.B.S. designed and oversaw the study; B.B., C.K., S.K., and P.B.S. analyzed the data; and B.B. and P.B.S. wrote the manuscript.

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Supplemental Material

Supplementary Figure S1, Supplementary Figure S2, Supplementary Figure S3, Supplementary Table 1, Supplementary Table 2

Supplemental Figure S1

(A-D) Examples of Staining Intensities. Immunohistochemistry was performed using antibodies against BCL-2 (clone 124, M0887, Agilent Technologies), BCL-XL (54H6, Cell Signaling Technologies) and MCL-1 (D5V5L, Cell Signaling Technologies) and was evaluated via H-score = [% of cells with 1+] + [% cells with 2+]x2 + [%cells with 3+]x3 (Supplemental Figure S1). (**A**) negative staining (ALCL, BCL2, reactive T-Cells are positive). (**B**) weakly intense staining (1+, Prostate Carcinoma, BCL-XL). (**C**) moderately intense staining (2+, Plasma Cell Myeloma, BCL2). (**D**) highly intense staining (3+, DLBCL, BCL2). (**E-G**) Staining in reactive lymph follicles. (**E**) BCL2. (**F**) BCL-XL. (**G**)MCL1; Scale Bar = 100 μm.

Supplemental Figure S2

(A-B) Correlation plots of MCL-1 and BCL-XL Scores versus AUC of Venetoclax in respective patients (n=34). **(C-D)** Correlation plots of MCL-1 versus BCL-2 and BCL-XL versus BCL-2 (n=34). **(E-F)** MCL1 score and BCL-XL score versus respective ex vivo response to Venetoclax stratified by indication.

Supplemental Figure S3

(A) Typical morphology of prolymphocytes in a blood sample of patient #1 before treatment start with venetoclax. Small to medium-sized cell with slightly irregular nuclear contours, that were CD4–/CD8+ with postthymic (TdT–, CD1a–) mature T-cell immunophenotype (CD2+, CD5+, CD7+, CD16–, CD56–) and regular expression of pan–T-cell markers and weak expression of CD25. Immunophenotype of patient #2's T-PLL cells was CD4weak/CD8+, TdT–, CD1a–, CD2+, CD5+, CD7+, CD16–, CD56–, CD3+, and weak CD25. **(B)** Patient #1: The pre-therapeutic transabdominal ultrasound examination reveals a marked splenomegaly with a maximum long-axis diameter of 24.7 cm. The contrast-enhanced CT (coronal section, rotated counter-clockwise for better comparison with the pre-therapeutic ultrasound) after 8 days of venetoclax treatment shows a more than 5 cm decrease of the splenic diameter (to 19.3 cm). **(C)** Patient #2: The pre-therapeutic contrast-enhanced CT (axial sections shown) shows pathologically enlarged supradiaphragmatic lymph nodes in both axillae and

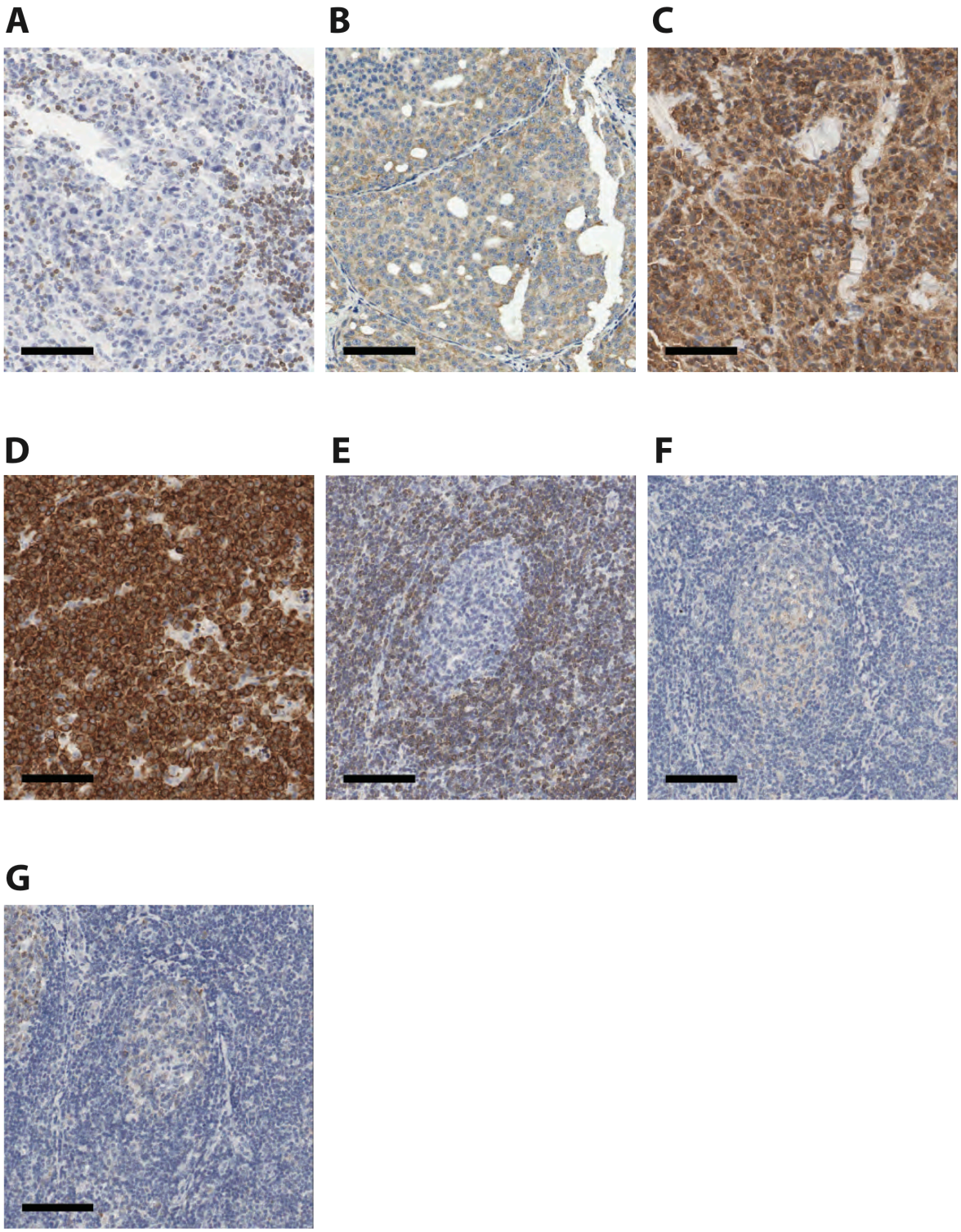
the mediastinum (blue arrows) as well as pathologically enlarged infradiaphragmatic lymph nodes in the mesentery (yellow arrows). After two months on venetoclax, there is complete regression of all pathologic lymph nodes in the axillae and the mesentery, and a size reduction of several mediastinal lymph nodes, in the contrast-enhanced follow-up CT **(D)** Barplot showing viability of samples used in Figure 2E after thawing as determined by CASY Cell Analyzer (OLS).

Supplementary Table 1 - Drug List Ex Vivo Screen

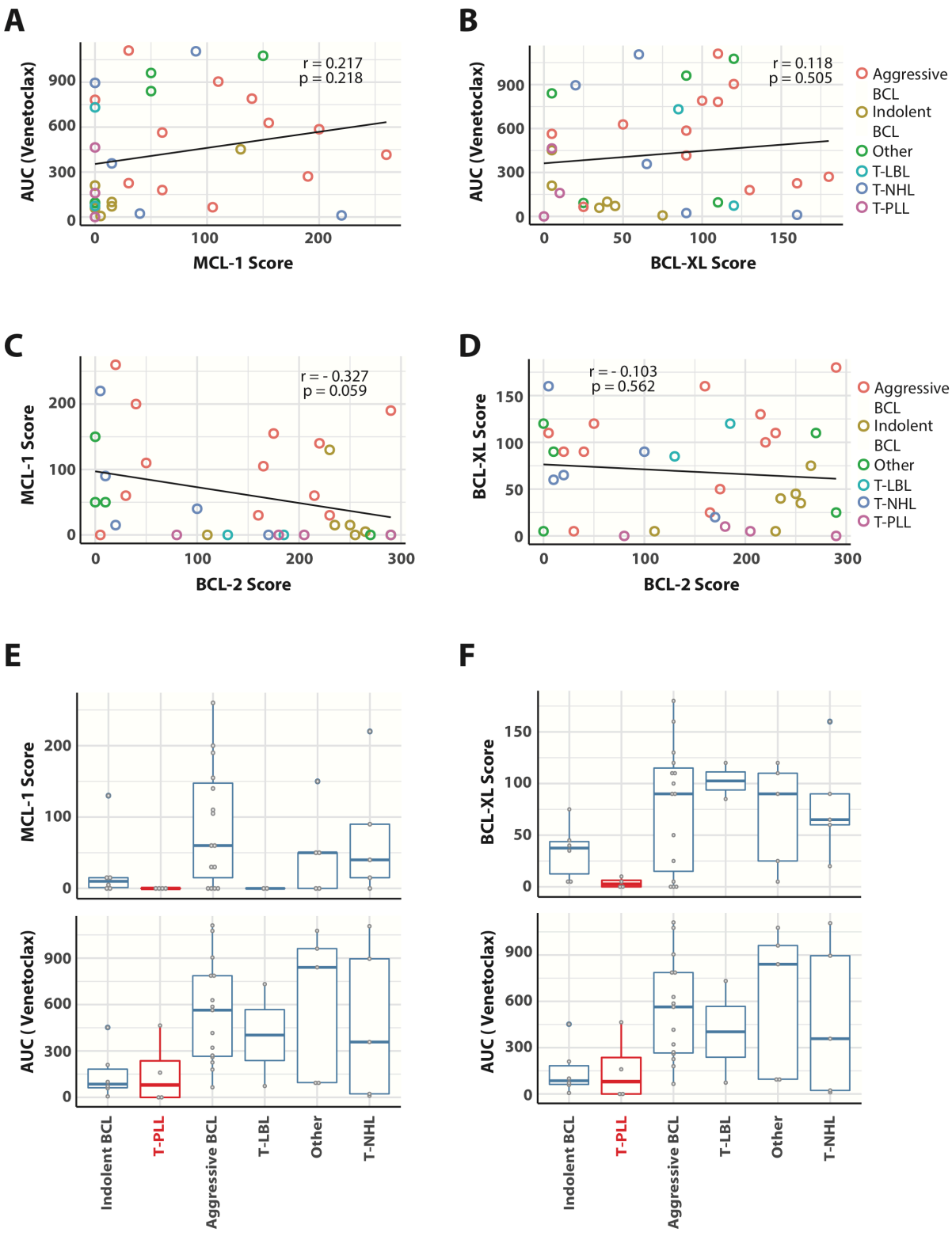
CompoundClass	CompoundName	Concentration 1 [uM]	Concentration 2 [uM]	Concentration 3 [uM]	Concentration 4 [uM]	Vendor(s)	FDA Approved
Alkylating agents	Altretamine	1	3	9	27	Enamine	Yes
Alkylating agents	Bendamustine	1	3	9	27	Selleckchem	Yes
Alkylating agents	Busulfan	1	3	9	27	Medchem Express	Yes
Alkylating agents	Carboplatin	1	3	9	27	Selleckchem	Yes
Alkylating agents	Carmustine	1	3	9	27	Sigma	Yes
Alkylating agents	Chlorambucil	0.533	1.599	4.797	14.391	Sigma	Yes
Alkylating agents	Cisplatin	1	3	9	27	Selleckchem	Yes
Alkylating agents	Cyclophosphamide	0.5	1.5	4.5	13.5	Sigma	Yes
Alkylating agents	Oxaliplatin	1	3	9	27	Selleckchem	Yes
Alkylating agents	Procarbazine	1	3	9	27	Medchem Express	Yes
Alkylating agents	Temozolomide	1	3	9	27	Medchem Express	Yes
Alkylating agents	Thiotepa	1	3	9	27	Sigma, Toronto Research Chemicals	Yes
Antimetabolites	5-Fluorouracil	1	3	9	27	Enamine	Yes
Antimetabolites	Capecitabine	1	3	9	27	Selleckchem	Yes
Antimetabolites	Clofarabine	0.005	0.015	0.045	0.135	LC Laboratories	Yes
Antimetabolites	Cytarabine	0.079057	0.237171	0.711513	2.134539	Enamine	Yes
Antimetabolites	Methotrexate	0.1852875	0.5558625	1.6675875	5.0027625	Medchem Express	Yes
Antimetabolites	Pentostatin	0.7345	2.2035	6.6105	19.8315	Sigma	Yes
Antimetabolites	Pralatrexate	0.352755	1.058265	3.174795	9.524385	Selleckchem	Yes
Antimitotics	Docetaxel	0.0187785	0.0563355	0.1690065	0.5070195	Enamine	Yes
Antimitotics	Paclitaxel	0.032697	0.098091	0.294273	0.882819	Selleckchem	Yes
Antimitotics	Vinblastine Sulfate	0.036504	0.109512	0.328536	0.985608	LC Laboratories	Yes
Antimitotics	Vincristine	1	3	9	27	Selleckchem	Yes
BH3 Mimetic	ABT-199	0.5	1.5	4.5	13.5	ChemieTek	No
Epigenetic	5-Azacytidine	1	3	9	27	Medchem Express, Sigma	Yes
Epigenetic	EPZ-5676	0.5	1.5	4.5	13.5	ChemieTek	No
Epigenetic	EPZ-6438	0.5	1.5	4.5	13.5	ChemieTek	No
Epigenetic	GSK126	0.5	1.5	4.5	13.5	ChemieTek	No
Epigenetic	I-BET762	0.5	1.5	4.5	13.5	ChemieTek	No
Epigenetic	JQ1-(+)	0.48	1.44	4.32	12.96	Medchem Express, Cayman	No
Epigenetic	ORY1001	0.5	1.5	4.5	13.5	Selenachem	No
Epigenetic	Romidepsin	0.00001947	0.00005841	0.00017523	5.26E-04	Carbosynth, ChemieTek, Selleckchem	Yes
Epigenetic	Vorinostat	0.19445	0.58335	1.75005	5.25015	Enamine	Yes
Hormone inhibitors	Abiraterone Acetate	1	3	9	27	Medchem Express	Yes
Hormone inhibitors	Aminoglutethimide	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Anastrozole	1	3	9	27	Medchem Express	Yes
Hormone inhibitors	Bicalutamide	1	3	9	27	Sigma, Tocris	Yes
Hormone inhibitors	Enzalutamide	1	3	9	27	Medchem Express	Yes
Hormone inhibitors	Exemestane	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Flutamide	1	3	9	27	Tocris	Yes
Hormone inhibitors	Fulvestrant	1	3	9	27	Medchem Express	Yes
Hormone inhibitors	Lanreotide Acetate	1	3	9	27	Sigma	Yes
Hormone inhibitors	Letrozole	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Leuprolide	1	3	9	27	Medchem Express, Sigma	Yes
Hormone inhibitors	Megestrol	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Nilutamide	1	3	9	27	Sigma, Tocris	Yes
Hormone inhibitors	Raloxifene	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Tamoxifen	1	3	9	27	Medchem Express	Yes
Hormone inhibitors	Toremifene	1	3	9	27	Selleckchem	Yes
Hormone inhibitors	Triptorelin	1	3	9	27	Selleckchem	Yes
Immunomodulators	Alitretinoin	1	3	9	27	Sigma, Cayman	Yes
Immunomodulators	Bexarotene	1	3	9	27	Selleckchem	Yes
Immunomodulators	Lenalidomide	0.208325	0.624975	1.874925	5.624775	Selleckchem	Yes
Immunomodulators	Prelone	0.058775	0.176325	0.528975	1.586925	Sigma	Yes
Immunomodulators	Thalidomide	0.4701375	1.4104125	4.2312375	12.6937125	Enamine	Yes
Immunomodulators	Tretinoin	1	3	9	27	Selleckchem	Yes
Kinase inhibitors	Afatinib	0.5	1.5	4.5	13.5	Medchem Express	Yes
Kinase inhibitors	Alisertib	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Axitinib	1	3	9	27	Selleckchem	Yes
Kinase inhibitors	Bafetinib	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Bosutinib	0.5	1.5	4.5	13.5	LC Laboratories	Yes
Kinase inhibitors	Cabozantinib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Cediranib	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Ceritinib	0.5	1.5	4.5	13.5	Medchem Express	Yes
Kinase inhibitors	Crizotinib	1	3	9	27	Medchem Express, Sigma	Yes
Kinase inhibitors	Dasatinib	0.001069803	0.00320941	0.009628231	2.89E-02	Cayman	Yes
Kinase inhibitors	Erlotinib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Gefitinib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Ibrutinib	0.5	1.5	4.5	13.5	ChemieTek	Yes
Kinase inhibitors	Idelalisib	0.5	1.5	4.5	13.5	ChemieTek	Yes
Kinase inhibitors	Imatinib	0.63585	1.90755	5.72265	17.16795	ChemieTek	Yes
Kinase inhibitors	IPI-145	0.5	1.5	4.5	13.5	ChemieTek	No
Kinase inhibitors	Lapatinib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Lestaurtinib	0.5	1.5	4.5	13.5	LC Laboratories	Orphan
Kinase inhibitors	Masitinib	0.5	1.5	4.5	13.5	LC Laboratories	Orphan
Kinase inhibitors	MLN2480	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	MLN4924	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Motesanib	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Neratinib	0.5	1.5	4.5	13.5	Abmole, Medchem Express	No
Kinase inhibitors	Nilotinib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Olaparib	0.5	1.5	4.5	13.5	LC Laboratories	Yes
Kinase inhibitors	Pacritinib	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Pazopanib	0.57275	1.71825	5.15475	15.46425	Selleckchem	Yes
Kinase inhibitors	Ponatinib	0.128975	0.386925	1.160775	3.482325	LC Laboratories	Yes
Kinase inhibitors	Regorafenib	1	3	9	27	Medchem Express	Yes
Kinase inhibitors	Ruxolitinib	1	3	9	27	Cayman, LC Laboratories	Yes
Kinase inhibitors	Semaxanib	0.5	1.5	4.5	13.5	Selleckchem	No
Kinase inhibitors	Sorafenib	0.6105	1.8315	5.4945	16.4835	Medchem Express	Yes
Kinase inhibitors	Sunitinib	0.301	0.903	2.709	8.127	Selleckchem	Yes
Kinase inhibitors	TAK-733	0.5	1.5	4.5	13.5	Medchem Express	No
Kinase inhibitors	Trametinib	0.5	1.5	4.5	13.5	LC Laboratories	Yes
Kinase inhibitors	Vandetanib	1	3	9	27	Selleckchem	Yes
Kinase inhibitors	Veliparib	0.5	1.5	4.5	13.5	Selleckchem	Orphan
Kinase inhibitors	Vemurafenib	1	3	9	27	LC Laboratories	Yes
iscellaneous antineoplasti	Hydroxyurea	1	3	9	27	Enamine	Yes
iscellaneous antineoplasti	Pixantrone	0.5	1.5	4.5	13.5	Medchem Express	Yes
Proteasome inhibitor	Bortezomib	0.0002975	0.0008925	0.0026775	8.03E-03	boratories, Medchem Express, Selleck	Yes
Proteasome inhibitor	Carfilzomib	0.0000585	0.0001755	0.0005265	1.58E-03	LC Laboratories	Yes
Proteasome inhibitor	Ixazomib	0.5	1.5	4.5	13.5	Medchem Express	Yes
Rapalogs	Everolimus	1	3	9	27	LC Laboratories, Medchem Express	Yes
Rapalogs	MLN0128	0.5	1.5	4.5	13.5	Selleckchem	No
Rapalogs	Temsirolimus	1	3	9	27	LC Laboratories, Selleckchem	Yes
Smoothened inhibitors	Vismodegib	1	3	9	27	Medchem Express	Yes
Topoisomerase inhibitors	Doxorubicin	0.046075	0.138225	0.414675	1.244025	LC Laboratories	Yes
Topoisomerase inhibitors	Irinotecan	1	3	9	27	Medchem Express	Yes
Topoisomerase inhibitors	Mitoxantrone	0.00572	0.01716	0.05148	0.15444	Enamine	Yes

Supplementary Table 2 - Patient Sample List (Ex Vivo Screen and TMA)

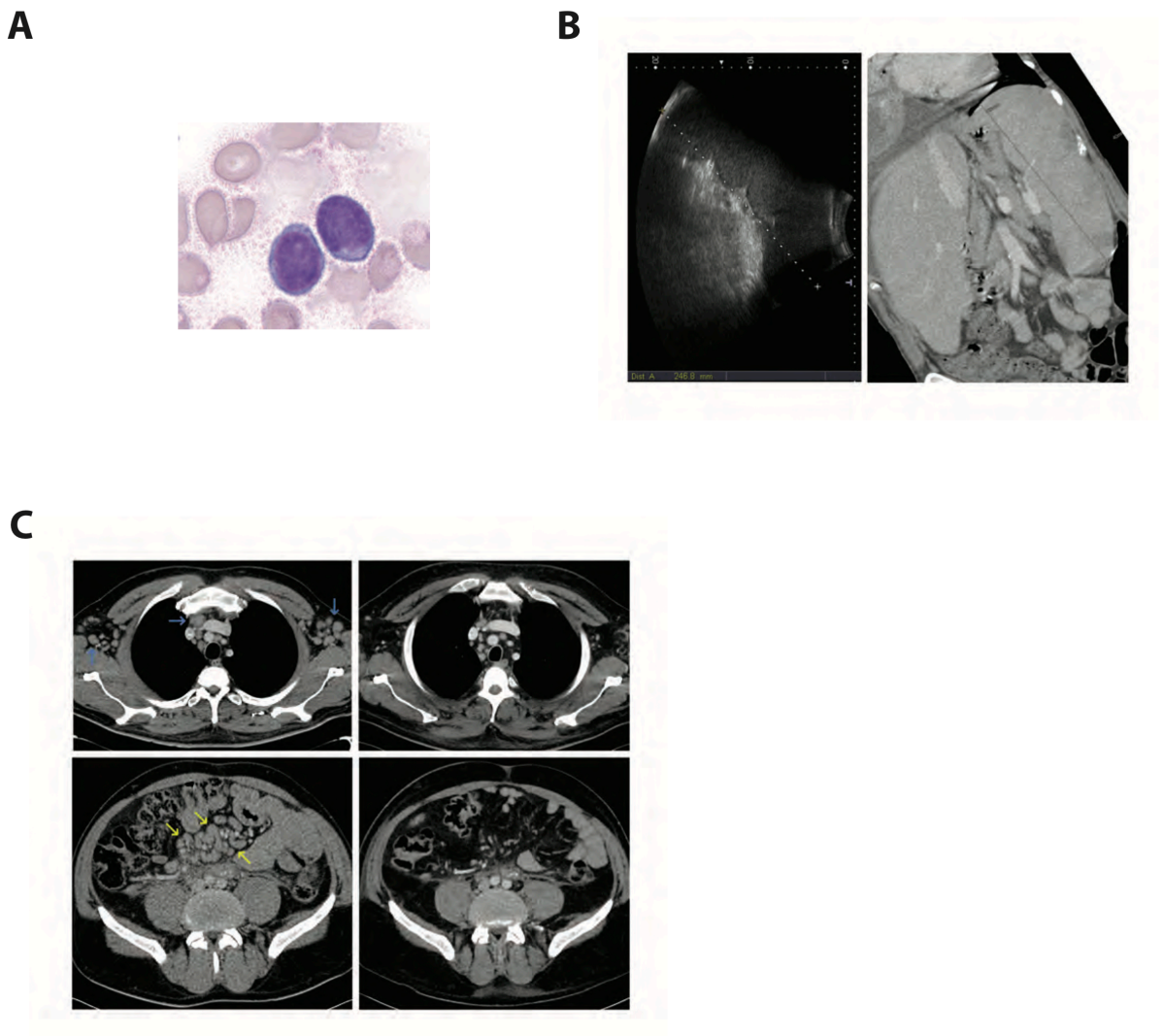
Sample ID	Diagnostic Group	Diagnosis	Tissue Source	TMA
1	T-NHL	T large granular lymphocyte leukemia	bone marrow	No
2	aggressive B-Cell lymphoma	mixed lymphoma Hodgkin's Lymphoma / prim. mediastinal B-NHL	lymph node	Yes
3	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, ABC, DPES2	lymph node	Yes
4	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, leg type, ABC	lymph node	Yes
5	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, DPES 1 (bcl2)	lymph node	No
6	T-LBL	T-lymphoblastic leukemia/lymphoma	lymph node	Yes
7	T-LBL	T-lymphoblastic leukemia/lymphoma	lymph node	Yes
8	indolent B-cell lymphoma	Follicular Lymphoma low grade	lymph node	Yes
9	CLL	Chronic lymphocytic leukemia	peripheral blood	No
10	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, ABC, DPES2	lymph node	Yes
11	AML	Acute myloid leukemia	peripheral blood	No
12	B-ALL	Acute lymphatic leukemia	peripheral blood	No
13	indolent B-cell lymphoma	Follicular Lymphoma low grade	lymph node	Yes
14	T-PLL	T-prolympholytic leukemia	peripheral blood	Yes
15	indolent B-cell lymphoma	Follicular Lymphoma	lymph node	No
16	T-PLL	T-prolympholytic leukemia	peripheral blood	Yes
17	AML	Acute myloid leukemia	peripheral blood	No
18	AML	Acute myloid leukemia	peripheral blood	No
19	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, EBV associated, ABC, DPES 1 (c-myc)	lymph node	Yes
20	AML	Acute myloid leukemia	peripheral blood	No
21	T-NHL	Anaplastic large cell lymphoma, ALK positive	lymph node	Yes
22	AML	Acute myloid leukemia	peripheral blood	No
23	AML	Acute myloid leukemia	peripheral blood	No
24	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, GCB, DPES2	lymph node	Yes
25	AML	Acute myloid leukemia	peripheral blood	No
26	AML	Acute myloid leukemia	peripheral blood	No
27	Other	Adenocarcinoma, colorectal	lymph node	No
28	Other	Adenocarcinoma of Prostate, cribriform	lymph node	Yes
29	AML	Acute myloid leukemia	peripheral blood	No
30	indolent B-cell lymphoma	Follicular Lymphoma	lymph node	No
31	aggressive B-Cell lymphoma	aggressive B Cell Lymphoma, transformed from indolent B-NHL	lymph node	Yes
32	AML	Acute myloid leukemia	peripheral blood	No
33	Other	Adenocarcinoma, mucinous	lymph node	Yes
34	T-NHL	Composite Lymphoma	lymph node	Yes
35	AML	Acute myloid leukemia	peripheral blood	No
36	AML	Acute myloid leukemia	peripheral blood	No
37	AML	Acute myloid leukemia	peripheral blood	No
38	AML	Acute myloid leukemia	peripheral blood	No
39	indolent B-cell lymphoma	Follicular Lymphoma low grade	lymph node	Yes
40	T-NHL	Mycosis fungoides	lymph node	No
41	AML	Acute myloid leukemia	peripheral blood	No
42	AML	Acute myloid leukemia	peripheral blood	No
43	AML	Acute myloid leukemia	peripheral blood	No
44	T-NHL	nodal peripheral T-cell lymphoma, follicular T-cell phenotype	lymph node	Yes
45	indolent B-cell lymphoma	Plasma cell myeloma	bone marrow	Yes
46	B-ALL	B-lymphoblastic leukemia/lymphoma	peripheral blood	No
47	AML	Acute myloid leukemia	peripheral blood	No
48	AML	Acute myloid leukemia	peripheral blood	No
49	AML	Acute myloid leukemia	peripheral blood	No
50	AML	Acute myloid leukemia	peripheral blood	No
51	aggressive B-Cell lymphoma	T- and histiocyte rich B-cell lymphoma	lymph node	Yes
52	AML	Acute myloid leukemia	peripheral blood	No
53	AML	Acute myloid leukemia	peripheral blood	No
54	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, CD5+, anaplastic	lymph node	Yes
55	AML	Acute myloid leukemia	peripheral blood	No
56	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS	lymph node	No
57	T-NHL	hepatosplenic gamma delta T-cell lymphoma	spleen	Yes
58	T-PLL	T-prolympholytic leukemia	peripheral blood	Yes
59	AML	Acute myloid leukemia	peripheral blood	No
60	indolent B-cell lymphoma	Mantle cell lymphoma	lymph node	Yes
61	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, Richter Transformation from CLL	lymph node	Yes
62	AML	Acute myloid leukemia	peripheral blood	No
63	T-NHL	Peripheral T-cell lymphoma, follicular variant	lymph node	No
64	AML	secondary acute myloid leukemia	peripheral blood	No
65	Other	Splenic diffuse red pulp small B-cell lymphoma	spleen	No
66	aggressive B-Cell lymphoma	T- and histiocyte rich B-cell lymphoma	lymph node	Yes
67	T-LBL	T-lymphoblastic leukemia/lymphoma	lymph node	No
68	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, ABC	lymph node	Yes
69	Other	Nodular lymphocyte predominant Hodgkin's lymphoma	lymph node	Yes
70	AML	Acute myloid leukemia	peripheral blood	No
71	Other	Squamous cell carcinoma G3	lymph node	Yes
72	AML	Acute myloid leukemia	peripheral blood	No
73	AML	Acute myloid leukemia	peripheral blood	No
74	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, GCB, DPES1 (bcl2)	lymph node	Yes
75	AML	Acute myloid leukemia	peripheral blood	No
76	Other	Blastic plasmacytic dentritic cell neoplasia	lymph node	Yes
77	indolent B-cell lymphoma	Follicular Lymphoma low grade	lymph node	Yes
78	indolent B-cell lymphoma	Mantle cell lymphoma	lymph node	Yes
79	AML	Acute myloid leukemia	peripheral blood	No
80	T-PLL	T-prolympholytic leukemia	peripheral blood	Yes
81	AML	Acute myloid leukemia	peripheral blood	No
82	aggressive B-Cell lymphoma	Diffuse large B-cell lymphoma, NOS, ABC, DPES 0	lymph node	Yes
83	T-NHL	Anaplastic large cell lymphoma, ALK positive	lymph node	Yes
84	AML	Acute myloid leukemia	peripheral blood	No
85	AML	Acute myloid leukemia	peripheral blood	No
86	indolent B-cell lymphoma	Follicular Lymphoma low grade	lymph node	No



Boidol et al. Supplemental Figure S1



Boidol et al. Supplemental Figure S2



Boidol et al. Supplemental Figure S3

Additional outcomes

After having established the BCL-2-inhibitor Venetoclax as a small molecule that triggers response in T-PLL patient samples as well as in the clinical treatment of the disease, we have screened another cohort of patients *ex vivo* to identify drugs that could act synergistically in concert with Venetoclax. T-PLL samples from 8 patients were thawed and seeded on drug plates containing Venetoclax and 14 additional compounds either alone or in combination with Venetoclax at 4 different concentrations. From the 8 patients, the results of one sample had to be excluded from the analysis due to high noise (T-PLL #2). The remaining patient samples showed excellent comparability across all drug combinations and the average of all patients for each single drug combination showed similar effects as the individual patient response alone, speaking for drug response findings that are less prone to be affected by the likely heterogeneous makeup of the patient cohort (Figure 4).

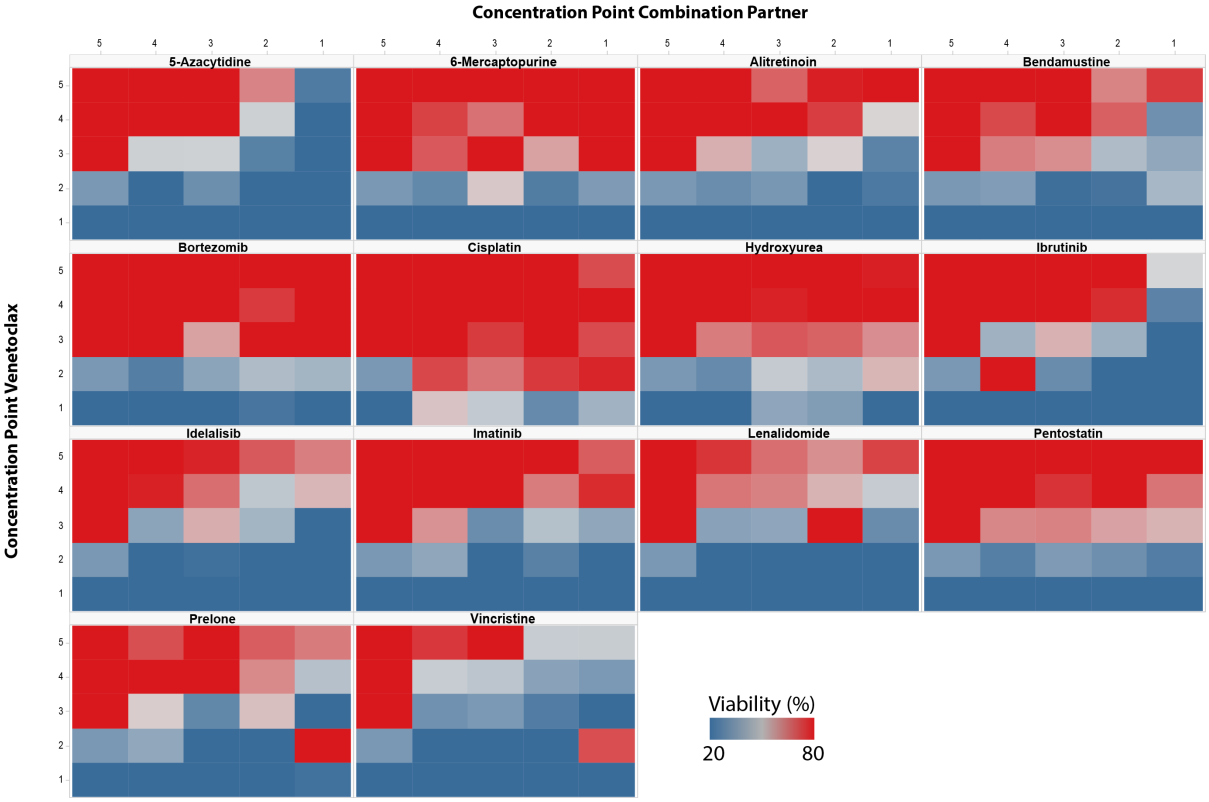


Figure 4 Average viability across all patients after ex vivo treatment with drug combination. *14 combination partners alone or together with Venetoclax were screened in T-PLL patient samples. Drug response across the patient cohort was highly similar and the average of the drug effects over all patients is therefore shown in a single heatmap. Concentrations can be found in the material and methods section of this thesis.*

To discriminate between additive and synergistic effects on T-PLL cells upon exposure to drug combinations, we have calculated the respective Bliss scores for every concentration point in the individual biopsies using the single drugs and compared it to the fractional inhibition seen when these drugs were combined (Bliss, 1939; Licciardello *et al*, 2017).

Positive deviations from this score denote a synergistic effect while negative deviations represent antagonistic activity of the drug combination. The results were averaged over all drug concentration points and visualized in a heatmap (Figure 5). Hierarchical clustering revealed 6 drugs that exert synergistic effects in T-PLL patient samples with Venetoclax.

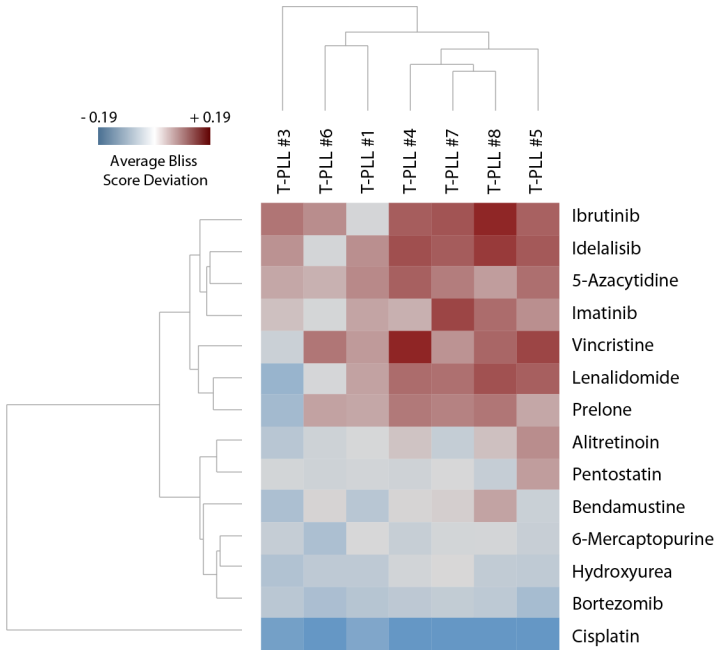


Figure 5 Average Bliss score deviation across all concentration points tested. *7 samples were screened with Venetoclax plus one of the 14 combination partners. Color corresponds to average deviation from Bliss score averaged over all concentration points tested.*

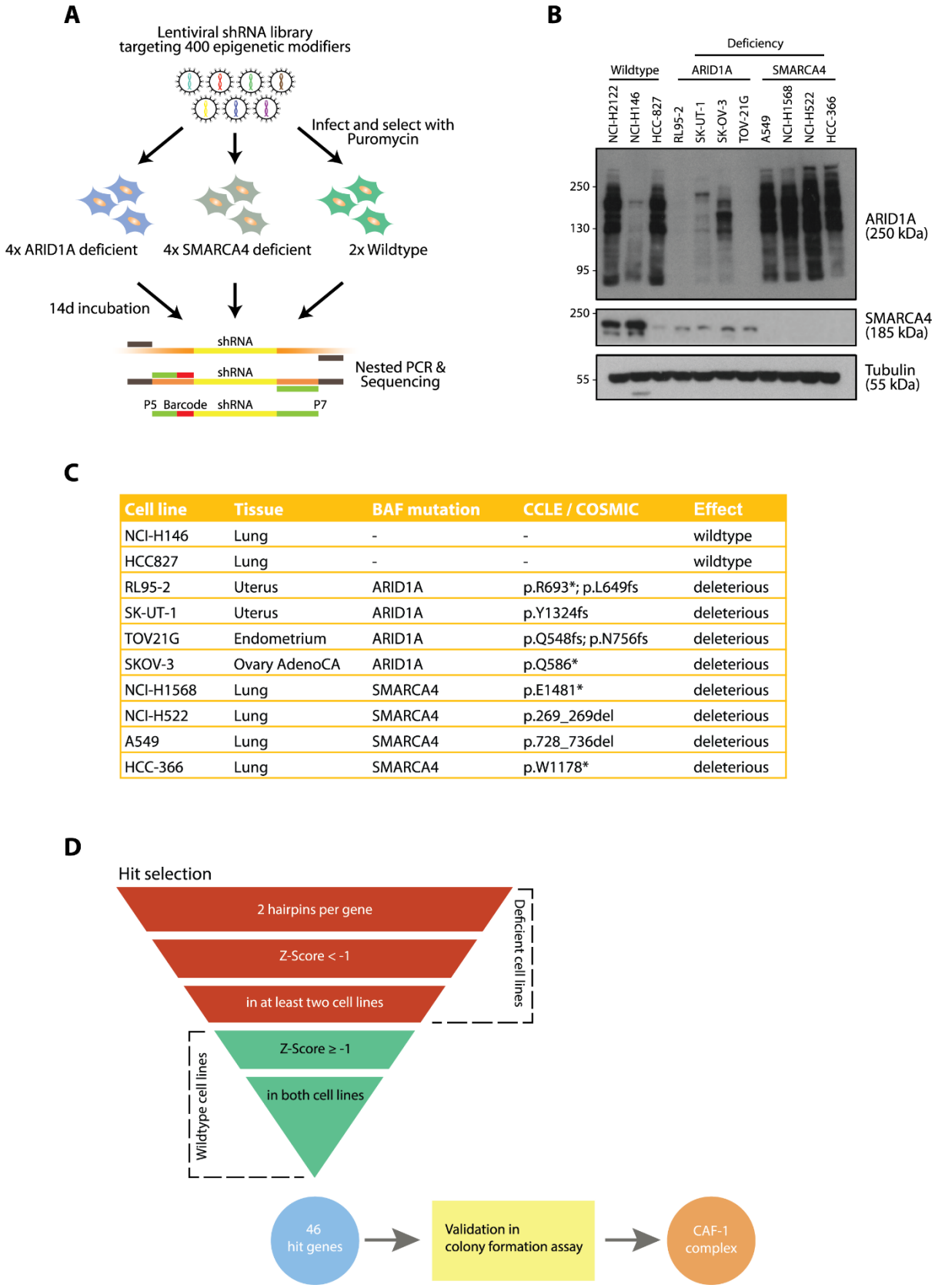
Various drugs in the panel show effects which enhance the activity of Venetoclax in the patient samples tested. Among those is the DNA methyltransferase inhibitor 5-azacytidine which has been shown to act synergistically with Venetoclax in samples taken from AML patients (Bogenberger *et al*, 2014). Surprisingly, Cisplatin showed dramatic antagonistic effects across all patients, i.e. the combination of both drugs results in weaker cell killing activity as either drug alone. It is worth mentioning that pure additivity of a combination partner with inhibition of BCL-2 might as well be regarded as beneficial for patients *in vivo*. However, drug synergism points towards a functional overlap of BCL-2 and the respective drug target of a synergistic combination partner.

These additional results show that BCL-2 inhibition by Venetoclax can be enhanced through treatment with further FDA-approved small molecules, constituting a potential combination treatment strategy for T-PLL patients. Moreover, antagonistic effects as seen in the case of Cisplatin must be closely taken into consideration when patients are treated with more than one drug.

2.2 Histone chaperone CAF-1 is a synthetic lethal target in ARID1A deficient cells

In order to identify vulnerabilities in BAF-deficient cancers, we have employed an epigenome-focused lentiviral RNAi library targeting 400 epigenetic modifiers with 4-5 short hairpin RNA constructs to screen cell lines deficient for either one of the two most frequently mutated BAF-subunit genes *ARID1A* and *SMARCA4*. As a control, two cell lines with wildtype complex were screened in parallel. Although the mutations found in CCLE and COSMIC databases were predicted to have deleterious effects on the respective proteins (Figure 6C), western blot analysis of the cell lines used in the screen together with a third wildtype, NCI-H2122 from lung origin, does not confirm this finding indubitably (Figure 6B). While cell lines A549, NCI-H1568, NCI-H522, and HCC-366 lack SMARCA4 and express ARID1A, the purported ARID1A deficient cell lines SK-UT-1 and SK-OV-3 still show expression of ARID1A isoforms smaller than 250 kDa. Wildtype cell line NCI-H146 also seems to lack the main isoform of ARID1A at 250 kDa although re-sequencing of this cell line did not detect mutations in the genomic regions of any BAF member. Cell lines were infected with a pool of shRNA's, incubated for 14 days and the abundance of each individual hairpin was determined using next generation sequencing (Figure 6A). Hit genes were detected as being targeted by at least two hairpins that lead to a Z-score of less than -1 in at least two deficient cell lines. Moreover, the same hairpins must not have a dramatic effect on cell viability (Z-score $\geq$ -1) in any of the two BAF-wildtype cell lines NCI-H146 and HCC-827 (Figure 6D). This selection process identified 46 candidate genes showing a growth impairment in BAF-deficient cell lines while sparing BAF-wildtype cell lines when knocked down by at least two hairpins in the screen. To confirm these effects, the identified candidate hairpins were used in colony formation assays (data not shown). Hairpins targeting *CHAF1A* and *CHAF1B*, two genes encoding subunits of the CAF-1 histone chaperone complex, could be validated in cell lines deficient for ARID1A or SMARCA4 but did not exhibit growth impairment in BAF-wildtype cell lines, in accordance with the original screening results (Figure 7A-F).

Interestingly, of four ARID1A-deficient cell lines in the screen, only the two cell lines that lack all isoforms of ARID1A are sensitive to *CHAF1A* or *CHAF1B* knockdown (Figure 7C and D), with RL95-2 being most vulnerable to knockdown of *CHAF1B* and TOV-21G showing reduced viability upon interference of both CAF-1 subunits. At the same time, none of the hit hairpins leads to substantial growth impairment in either of the wildtype cell lines, pointing towards CAF-1 subunits CHAF1A and CHAF1B being specific synthetic lethal targets in the absence of the most frequently mutated BAF-subunits.



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Cell line	Tissue	BAF mutation	CCLE / COSMIC	Effect
NCI-H146	Lung	-	-	wildtype
HCC827	Lung	-	-	wildtype
RL95-2	Uterus	ARID1A	p.R693*; p.L649fs	deleterious
SK-UT-1	Uterus	ARID1A	p.Y1324fs	deleterious
TOV21G	Endometrium	ARID1A	p.Q548fs; p.N756fs	deleterious
SKOV-3	Ovary AdenoCA	ARID1A	p.Q586*	deleterious
NCI-H1568	Lung	SMARCA4	p.E1481*	deleterious
NCI-H522	Lung	SMARCA4	p.269_269del	deleterious
A549	Lung	SMARCA4	p.728_736del	deleterious
HCC-366	Lung	SMARCA4	p.W1178*	deleterious

Figure 6 RNAi screen in BAF-deficient cell lines identifies CAF-1 complex as synthetic lethal target in BAF-mutant cancers. *(A) Depiction of screening concept. (B) Western blot of cell lines used in screen and BAF-wildtype cell line H2122 for comparison. (C) Overview of BAF mutations in cell lines used in screen. (D) Hit selection algorithm. Validation of candidate genes confirmed CAF-1 subunits CHAF1A and CHAF1B as synthetic lethal targets in BAF mutant cancers.*

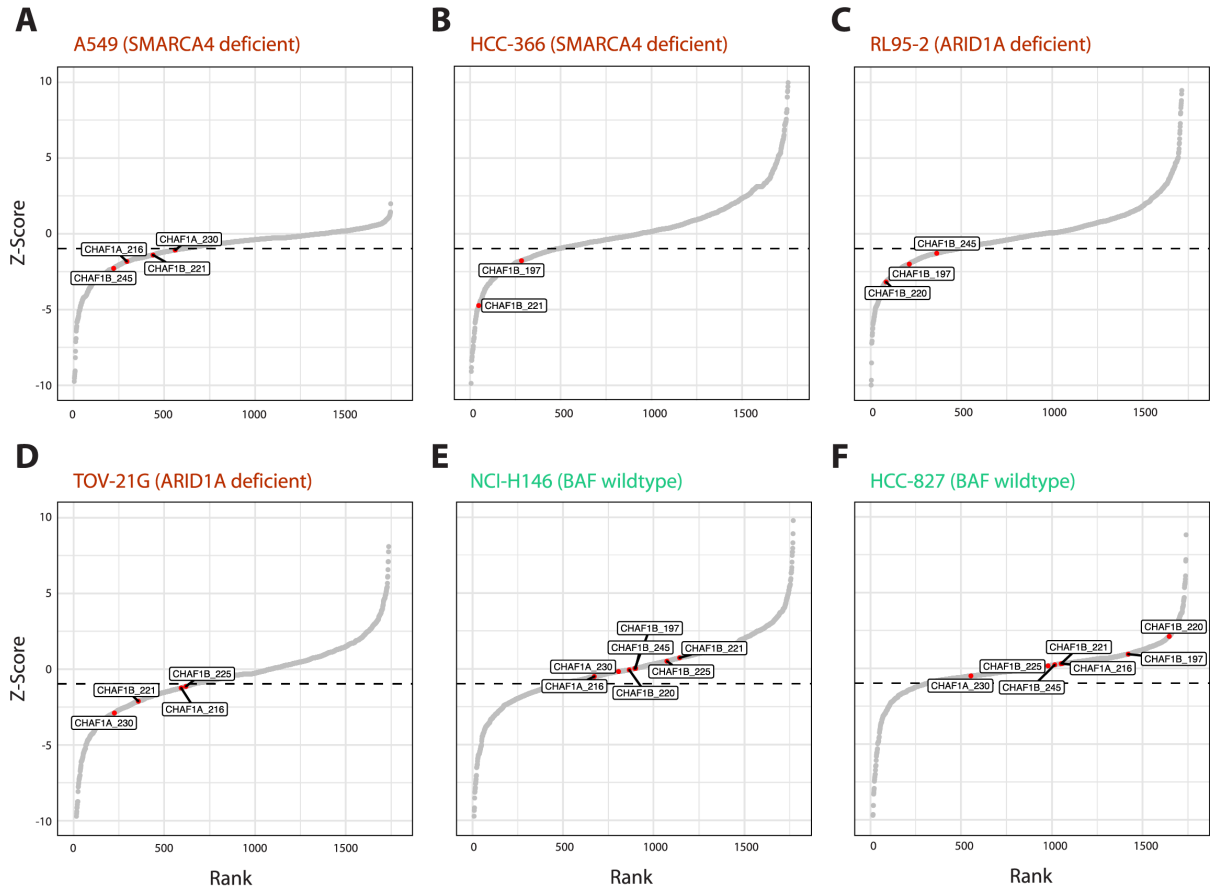

Figure 7 CHAF1A and CHAF1B hairpin performance in RNAi screen. *SMARCA4 deficient cell lines (A) A549 and (B) HCC-366 as well as ARID1A deficient cell lines (C) RL95-2 and (D) TOV-21G are sensitive to knockdown of CHAF1A or CHAF1B. BAF wildtype cell lines (E) NCI-H146 and (F) HCC-827 do not show growth impairment upon knockdown with these hairpins.*

Despite serving as an efficient discovery tool for RNAi screens, the genetic heterogeneity of these cell lines poses a challenge to further elucidating the interactions of BAF-complex members and the CAF-1 complex in regard of their synthetic lethal relationship. For this reason, we have made use of an isogenic HAP1 cell line pair in which the wildtype clone has no mutations in any of the BAF-complex members and the HAP1::ARID1A clone has been engineered to carry a 13 bp deletion in Exon 2 of *ARID1A*, leading to a full knockout (Figure 8B). A GFP competition assay was used to confirm the synthetic lethality of *CHAF1A* and *CHAF1B* in the *ARID1A* knockout clone (Figure 8A), a concept that we have successfully used for validation of synthetic lethalities previously (Licciardello *et al*, 2015). HAP1 wildtype cells were infected with a GFP construct, sorted, and mixed with HAP1::ARID1A cells. Upon lentiviral infection with a non-targeting control construct and constructs targeting *CHAF1A* or *CHAF1B*, cells were incubated for several days and FACS analysis revealed the abundance of GFP positive and negative cells over time in the knockdown fractions and in the control condition, allowing the calculation of relative *ARID1A* knockout cell abundance upon knockdown of synthetic lethal candidate genes. This approach controls the multiplicity of

infection (MOI) for both cell lines and facilitates the analysis of candidates that are otherwise essential for cell survival.

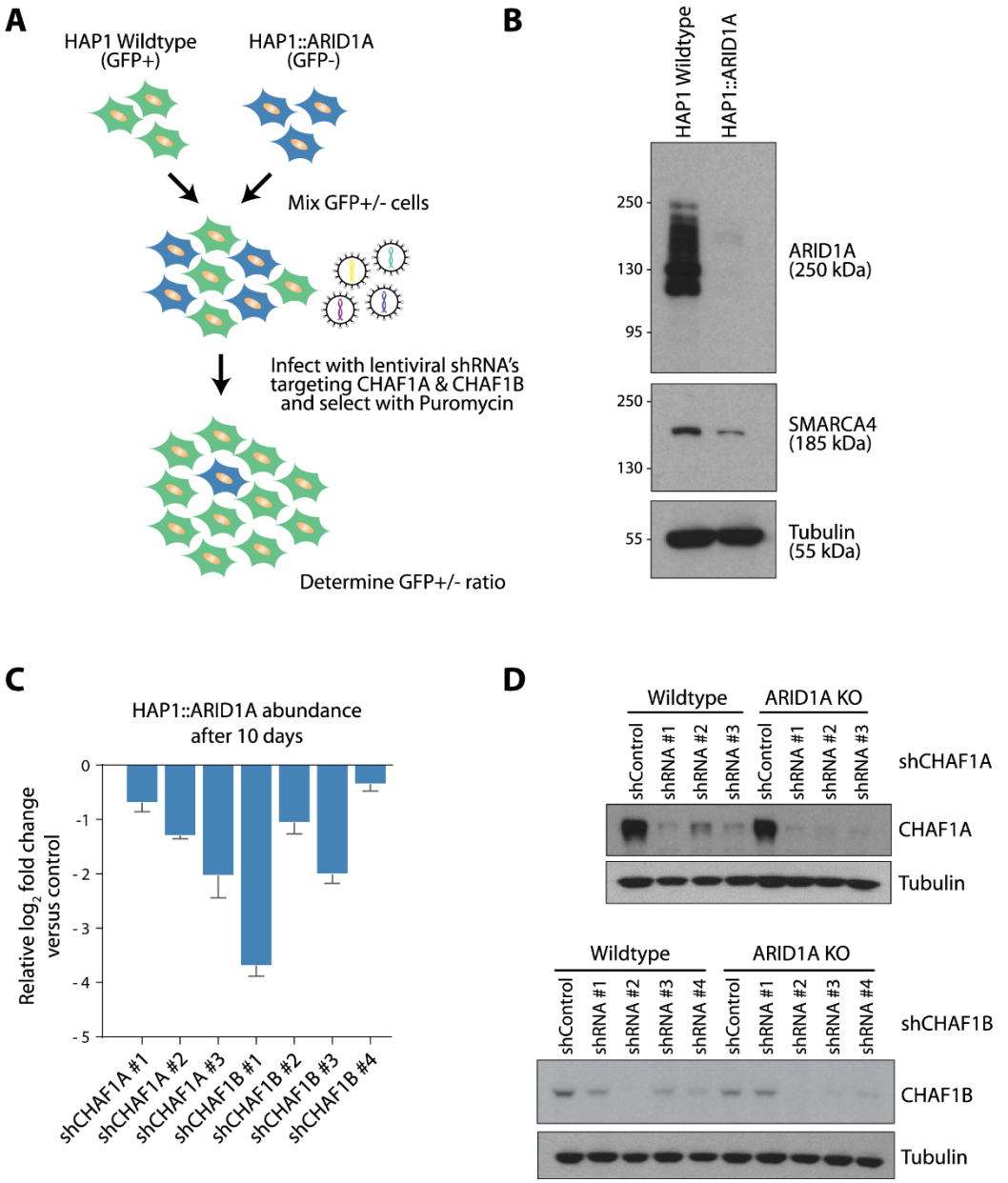

Figure 8 HAP1::ARID1A cells show relative growth impairment upon knockdown of CHAF1A and CHAF1B. *(A) Depiction of GFP competition assay. (B) Western blot confirming absence of ARID1A in HAP1::ARID1A knockout cells. (C) HAP1::ARID1A abundance 10 days post knockdown of CHAF1A and CHAF1B compared to HAP1 wildtype cells. Error bars represent standard deviation of triplicates. (D) Knockdown efficiency of hairpins used in GFP competition assay 3 days post infection.*

Knockdown of *CHAF1A* and *CHAF1B* leads to a reduction of HAP1::ARID1A cell abundance in the mix compared the control condition (Figure 8C). Although all of the hairpins employed in the assay resulted in an *ARID1A* knockout-specific growth impairment phenotype, the

magnitude of this effect did not correlate with the potency of the individual hairpins in reducing the respective protein levels (Figure 8D). This observation might be explained in part by the essentiality of CAF-1 for cell survival in HAP1 cells. A strong knockdown of either subunit will also interfere with viability of wildtype cells due to a narrow effective window, resulting in an equilibration of GFP positive and negative abundance in the cell mix and a less pronounced differential phenotype.

Although the utilization of short hairpin RNA constructs has facilitated the study of single genes and their role in cellular biology, unspecific binding to other mRNA's appears often and these off-target effects have the potential to distort results found through shRNA-mediated gene knockdown studies (Singh *et al*, 2011). To minimize off-targets, we have repeated the GFP competition assay and replaced shRNA's with pools of chemically modified siRNAs which have high silencing potential and very low off-target activity. Again, HAP1::ARID1A abundance is decreased significantly after silencing of *CHAF1A* or *CHAF1B* (Figure 9A and B), confirming CAF-1 as an ARID1A-specific vulnerability.

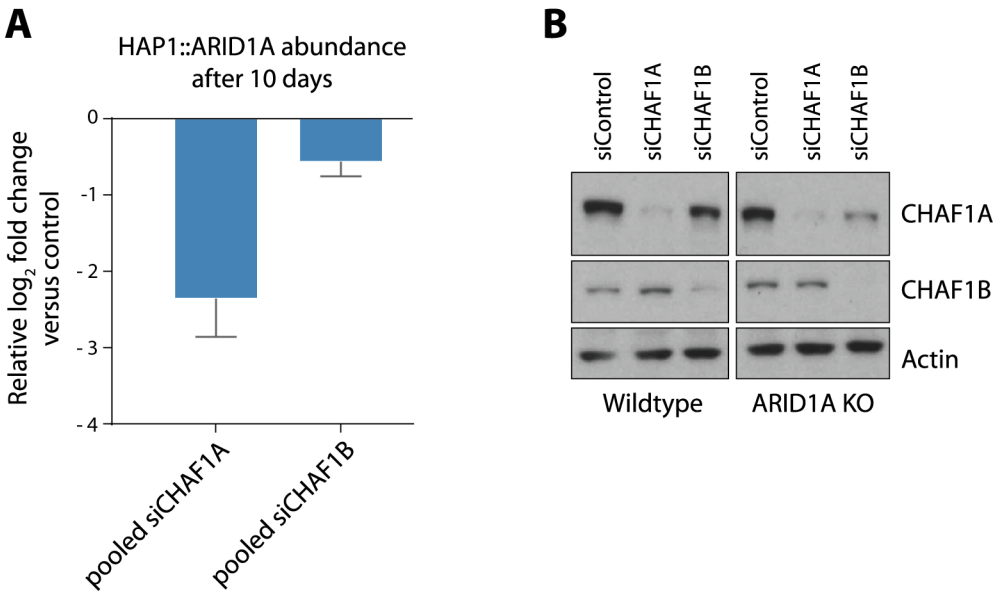

Figure 9 Pooled siRNA knockdown confirms CHAF1A and CHAF1B as synthetic lethal targets in ARID1A knockout cells. *(A) HAP1::ARID1A abundance 10 days post knockdown of CHAF1A and CHAF1B compared to HAP1 wildtype cells. Error bars represent standard deviation of triplicates. (B) Knockdown efficiency of hairpins used in GFP competition assay 3 days post transfection.*

Inducible knockdown constructs combine the knockdown efficiency of siRNA's with the prolonged reduction of target protein levels of shRNA's in a safe and easy fashion and constitute an even faster tool for further experiments. Therefore, we have generated HAP1 wildtype and HAP1::ARID1A cells carrying inducible hairpin constructs targeting *CHAF1A* and

a non-targeting control. Knockdown efficiency of these hairpins has been confirmed (Figure 10A) and relative, time-resolved reduction of *ARID1A* knockout cells in a GFP competition assay has been validated (Figure 10B).

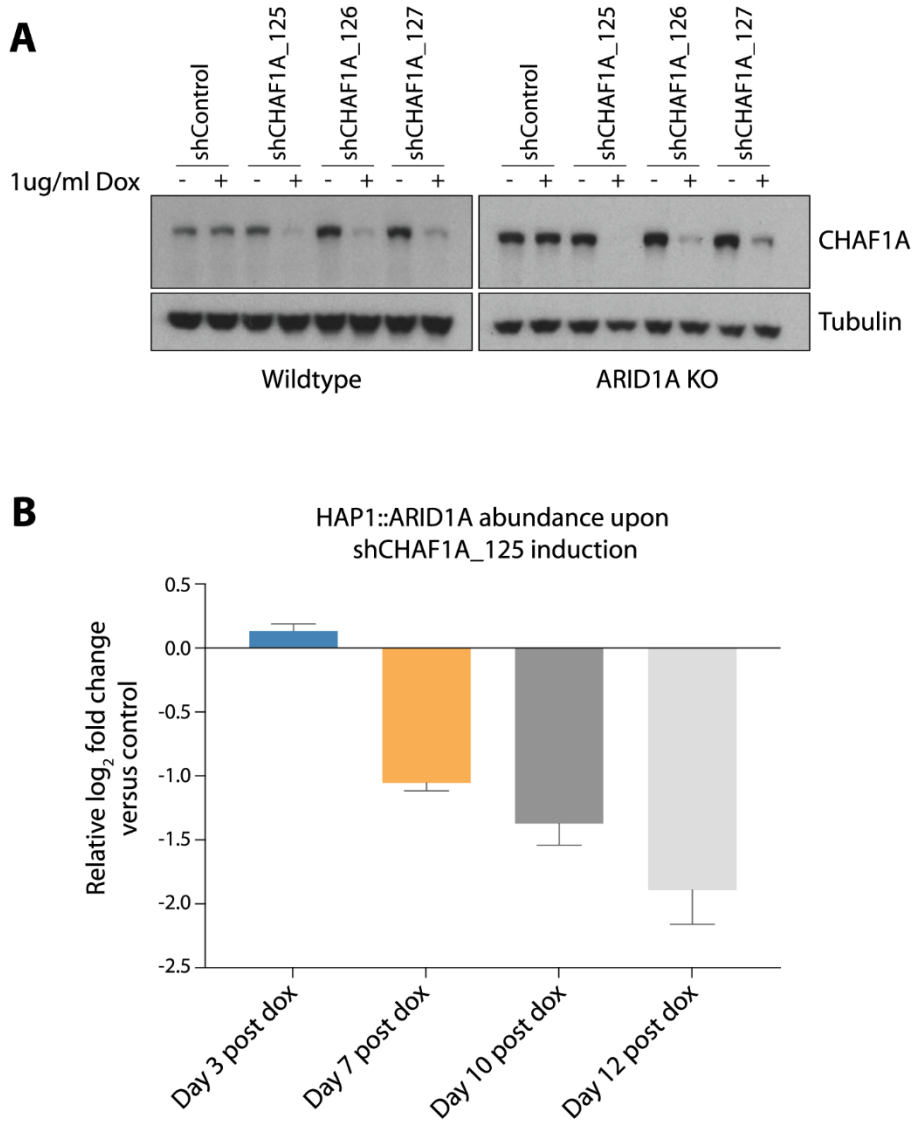

Figure 10 Doxycycline-inducible CHAF1A knockdown results in HAP1::ARID1A specific cell death. *(A) Western blot confirming knockdown efficiency of inducible hairpins upon induction with doxycycline (3 days post induction). (B) GFP competition assay of HAP1::ARID1A and HAP1 wildtype cells upon induction of shCHAF1A_125 in triplicate.*

Although various shRNA and chemically modified, target-specific siRNA constructs have been validated to show similar effects when knocking down *CHAF1A* or *CHAF1B*, the ultimate proof that the phenotype is not triggered by knockdown of an off-target would be the overexpression of a knockdown-resistant protein. We have tried to overexpress point-mutated *CHAF1A* and *CHAF1B* constructs in HAP1 cells to validate the on-target effects, however overexpression

did not work. While the respective tag was detectable on a protein level, we could not see any changes in CHAF1A or CHAF1B protein levels (data not shown). Another possibility to proof the specificity of these constructs encompasses the alteration of the endogenous locus of *CHAF1A* or *CHAF1B* at the target sequence of a hairpin of interest by introducing silent point mutations, i.e. bases that prohibit hairpin binding but code for a synonymous amino acid sequence so that the protein composition does not change (Figure 11A). The endogenous locus of *CHAF1A* in HAP1::ARID1A cells at the binding site of shCHAF1A_125 was cut using a sequence specific CRISPR construct and a point-mutated DNA template carrying silent alterations of the shCHAF1A_125 target sequence was co-transfected (Figure 11B).

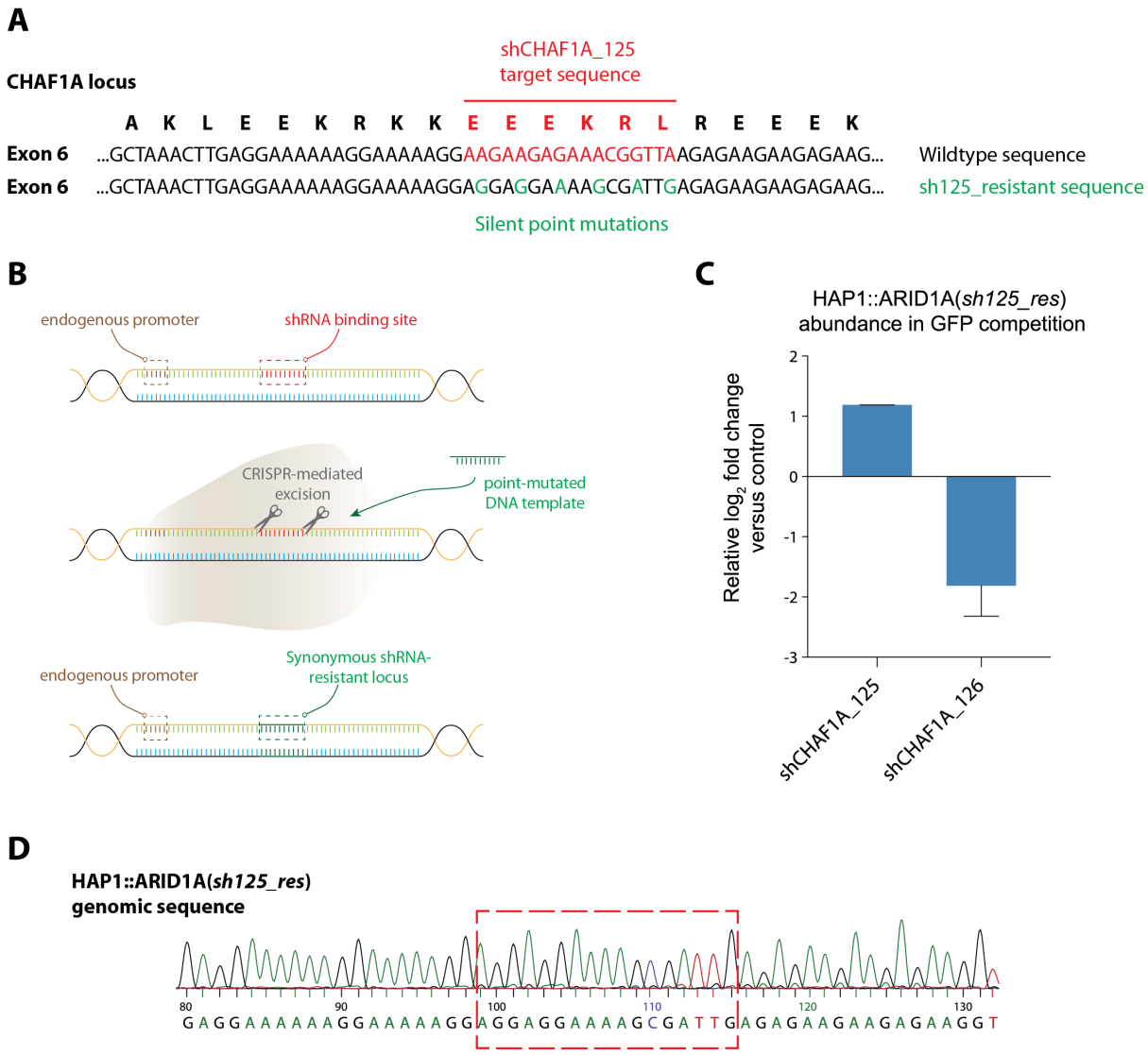

Figure 11 Homologous recombination-mediated introduction of silent point mutations confirms CHAF1A as phenotype-triggering target of shCHAF1A_125. *(A) Genomic sequence of CHAF1A. Original sequence with shCHAF1A_125 target sequence (red) and intended mutations (green). (B) Concept of homologous recombination strategy. (C) GFP competition assay of shCHAF1A_125 resistant HAP1::ARID1A clone 10 days post induction in duplicate. (D) Sanger sequencing confirmation of successful homologous recombination.*

Sanger sequencing confirmed the creation of a homozygous introduction of silent DNA alterations at this locus through homologous recombination using the provided DNA oligo as a template (Figure 11D). The GFP competition assay using HAP1 wildtype cells with a wildtype *CHAF1A* locus and HAP1::ARID1A(*shCHAF1A_125res*) clones revealed an increase in *ARID1A* knockout cell abundance when using shCHAF1A_125 but a decrease upon knockdown with shCHAF1A_126 (Figure 11C). The increase is explained by the general toxicity of CAF-1 knockdown in HAP1 cells. While the wildtype cells slowly undergo cell death, the *ARID1A* knockout cells are not affected by the hairpin. On the other side, knocking down the same gene *CHAF1A* with a different hairpin (shCHAF1A_126) decreases the viability of *ARID1A* knockout clones specifically. Rescuing the effects of hairpin shCHAF1A_125 using CRISPR-mediated homologous recombination of a point mutated DNA template shows the on-target activity of the CHAF1A hairpin and validates CAF-1 as a target protein complex in the absence of ARID1A.

To shed light onto the mechanistic interplay of ARID1A and CAF-1, we have performed transcriptional profiling and chromatin accessibility analysis. The loss of ARID1A in HAP1 cells leads to a significant deregulation of more than 600 genes, of which approximately half are upregulated and the other half are downregulated (Figure 12A). Feeding these genes into a gene set enrichment analysis identifies the terms “H3K27me3”, “EZH2”, and “SUZ12” at a high level of significance (Figure 12B). Interestingly, these terms are all associated with the functions of the Polycomb Repressive Complex 2 (PRC2), a histone methyltransferase which has been shown to antagonize the functions of the BAF complex (Kadoch & Crabtree, 2015) and Enhancer of Zeste Homolog 2 (EZH2), a subunit of the PRC2 complex that has been identified as a synthetic lethal target in *ARID1A* mutant cell lines (Bitler *et al*, 2015). Moreover, the vast majority of up- and downregulated genes are associated with PRC2 in certain ways (Figure 12C). Since analysis of CAF-1 knockdown in wildtype versus *ARID1A* knockout cells did not lead to a single set of candidate genes whose deregulation might be causative for the cell death phenotype seen in the RNAi screen and GFP competition assays, we moved to a more global analysis of a stepwise model of ARID1A loss and CAF-1 knockdown (Figure 12D and E). Specifically, we looked at gene deregulation after loss of ARID1A compared to the wildtype cell line (horizontal axis) and then analyzed how these genes change when we knock down *CHAF1A* or *CHAF1B* on top of the ARID1A null background (vertical axis). Surprisingly, genes that were deregulated into one direction by the loss of ARID1A showed the trend of going back to the opposite direction by knocking down *CHAF1A* or *CHAF1B*. This observation was also emphasized by analysis of nucleosome positioning on a global level (Figure 12F). Loss of ARID1A in HAP1 cells led to an increase in DNA fragment length, corresponding to an overall lower accessibility of DNA (Figure 12F last row).

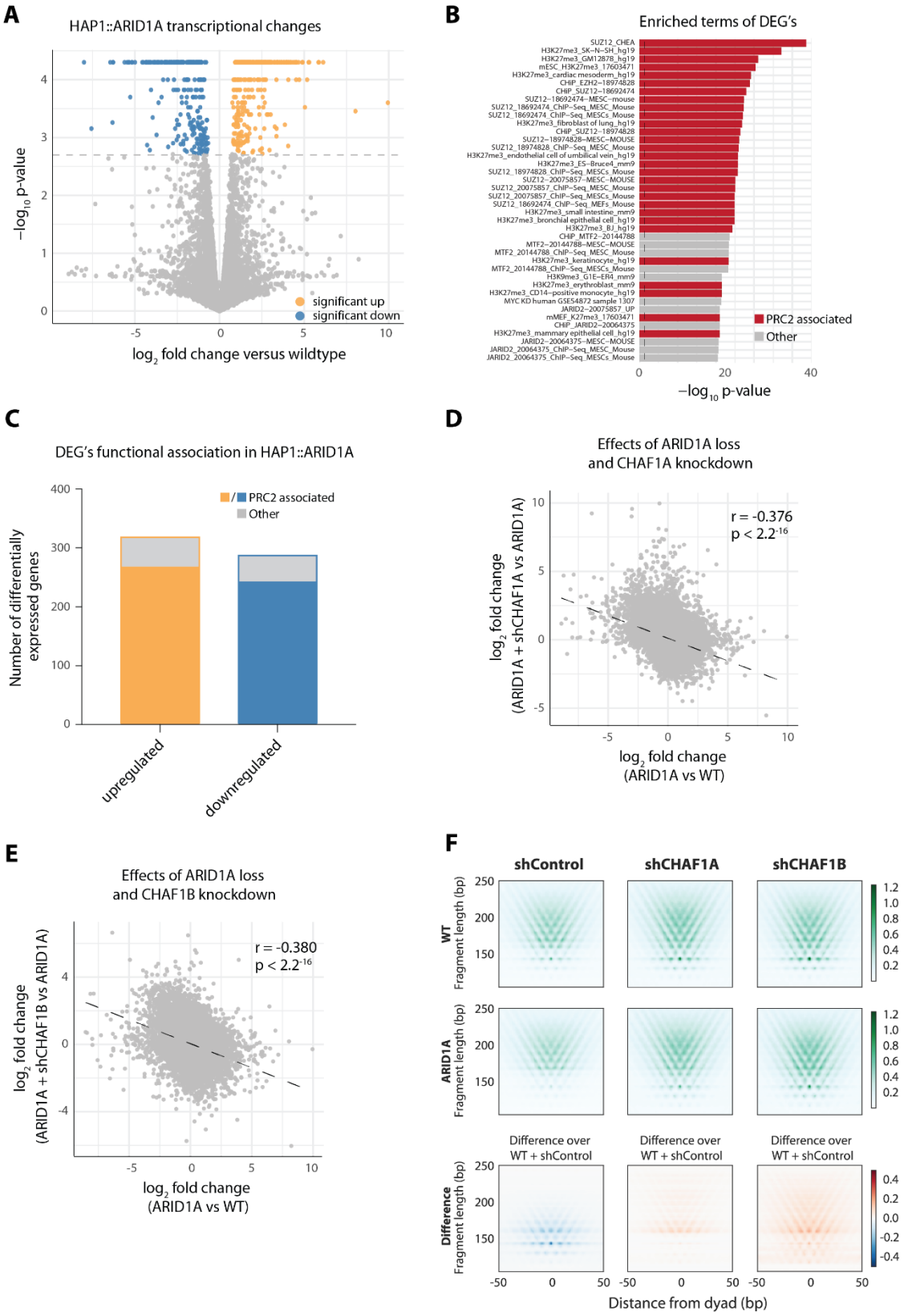

Figure 12 RNA-seq and ATAC-seq analysis of ARID1A loss and CHAF1A/CHAF1B knockdown. *(A) Volcano plot showing transcriptional changes in HAP1::ARID1A compared to wildtype. (B) Enriched gene set terms of differentially expressed genes (DEG's). (C) Functional association of DEG's. (D and E) Effects of ARID1A loss (horizontal axis) versus additional knockdown in HAP1::ARID1A cells (vertical axis). One representative hairpin per gene. (F) V-plots of nucleosome positioning in HAP1 wildtype and HAP1::ARID1A with and without knockdown of CHAF1A and CHAF1B. Lower panel shows differential nucleosome positioning of respective HAP1::ARID1A compared to HAP1 wildtype plus control hairpin (average of three hairpins per gene).*

However, knockdown of *CHAF1A* or *CHAF1B* resulted in an increased accessibility in the *ARID1A* knockout cells as compared to the wildtype, rescuing the effects caused by the loss of ARID1A (Figure 12F last row). These data show that the consequences of CAF-1 knockdown rescue parts of the transcriptional and chromatin accessibility phenotype triggered by loss of ARID1A on a global level.

Finally, to identify small molecules that enhance the effects of *CHAF1A* knockdown in *ARID1A* deficient HAP1 clones, HAP1 wildtype and HAP1::ARID1A cells carrying an inducible knockdown construct against *CHAF1A* or a non-targeting control were used for screening a library of more than 2,000 well annotated small molecules and compounds that have shown high toxicity in previous screens with other cell types at single concentrations.

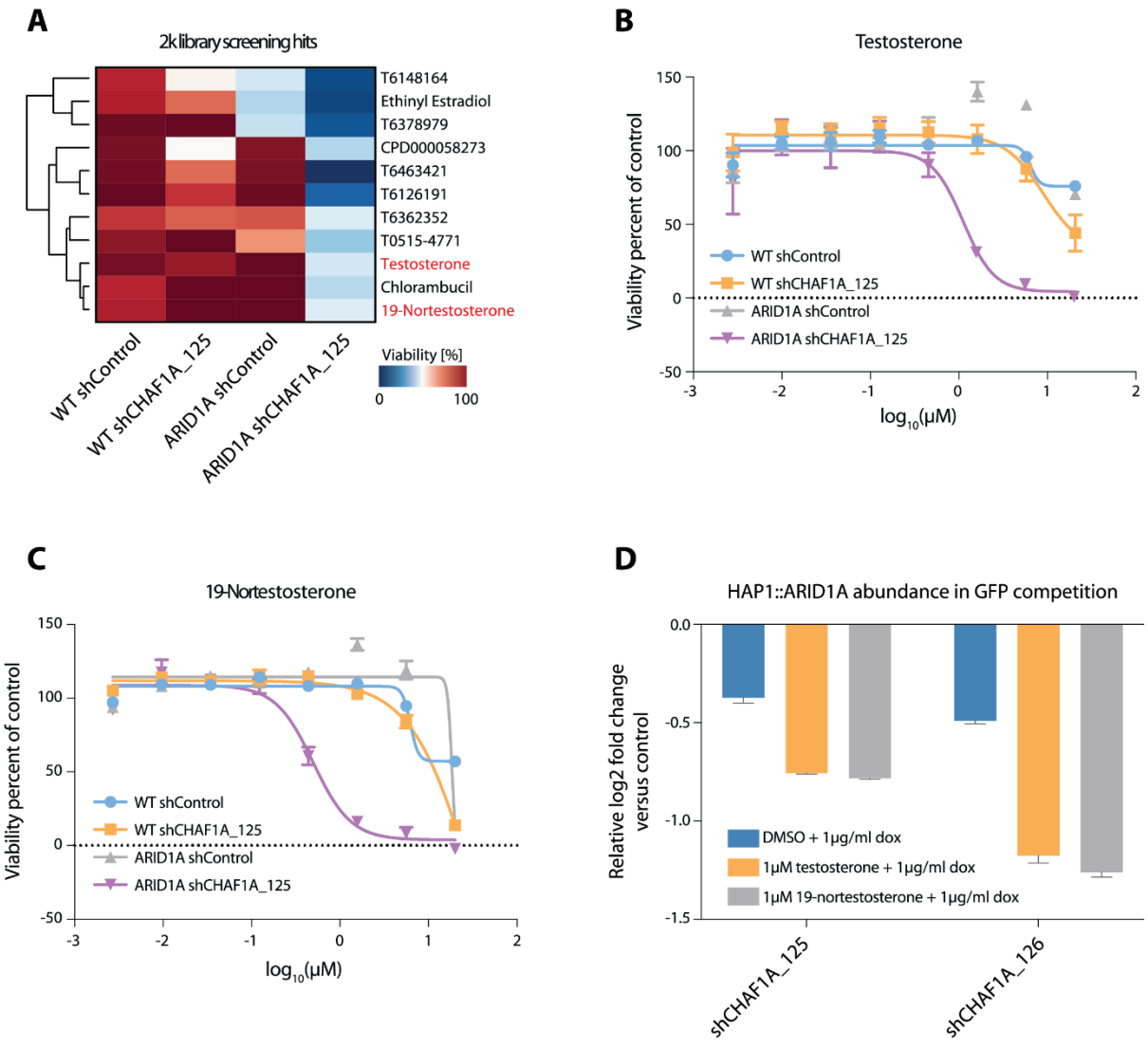

Figure 13 Drug screen in HAP1 wildtype and HAP1::ARID1A upon induction of CHAF1A knockdown. *(A) Heatmap depicting screening hits. (B and C) Dose response curves of testosterone and 19-nortestosterone in triplicate. (D) GFP competition assay of HAP1 wildtype and HAP1::ARID1A upon induction of knockdown and in the presence of 1 µM testosterone, 1 µM 19-nortestosterone, or DMSO as control, in duplicate.*

Both testosterone as well as the physiologically less common 19-nortestosterone were identified as drugs that specifically kill HAP1::ARID1A upon induction of *CHAF1A* knockdown with hairpin shCHAF1A_125 (Figure 13A). Dose response curves performed with all four cell lines revealed an IC_{50} of approximately 1 µM for both molecules in HAP1::ARID1A cells + shCHAF1A_125 while wildtype HAP1 and mock-infected HAP1::ARID1A cells show an IC_{50} of approximately 10-20 µM (Figure 13B and C). Similarly, the GFP competition assay with two hairpins against *CHAF1A* showed *ARID1A* knockout-specific growth impairment upon induction of the hairpins which was more than twice as dramatic when cell mixes were grown in the presence of 1 µM testosterone or 19-nortestosterone (Figure 13D).

In summary, an epigenome-focused RNAi screen in BAF-deficient cell lines has identified CAF-1 as a specific vulnerability in an ARID1A deficient background and these findings were validated in another cell line model. Mechanistically, the knockdown of CAF-1 reverts the transcriptional and chromatin accessibility phenotype that is caused by loss of ARID1A and makes cells sensitive to androgen receptor agonists testosterone and 19-nortestosterone. It remains to be seen, however, how the rescue of an ARID1A loss phenotype contributes to specific growth impairment and cell death. Moreover, the effects of testosterone might not only be caused by signaling via the androgen receptor but also by off-targets due to the high concentrations required for specific cell killing. Nevertheless, the histone chaperone complex CAF-1 represents a prime target in ARID1A-deficient cells and these findings can serve as a starting point for further drug development efforts.

3. Discussion

3.1 General discussion

The field of cancer research and cancer drug development has experienced dramatic revolutions during the past 120 years. Paul Ehrlich's "magic bullet" concept launched the quest for agents that would specifically target malignant cells while sparing healthy tissues. Systematic chemical screening as well as serendipitous discoveries yielded molecules that could be used in the treatment of cancer, even though the accompanying side-effects were, and still are, often a devastating price that needs to be paid for the treatment. The discovery of oncogenes and tumor suppressor genes was a hallmark in the molecular understanding of how tumors develop and allowed a systematic design of drugs that could block or even kill the malignantly deregulated cells. While the growing knowledge about oncogenes soon lead to new chemical entities for the fight against certain tumor types, tumor suppressor genes whose loss-of-function can only in the rarest cases be reverted by small molecules, remained a challenge to deal with. Ground-breaking concepts like oncogene-addiction have contributed immensely to a personalization of cancer medicine. All these findings have resulted in the advent of small molecules and biologicals that could be used, for the first time, to treat cancers effectively and with little adverse events based on the genetic makeup of a patient's tumor. One cannot appreciate enough the scientific leaps that were taken in the past decades and the speed at which research has moved towards improving and dealing with the ever-increasing rates of cancer incidence worldwide.

Despite of the seminal regulatory approvals of the past decades and the growing number of new chemical and biological entities targeting oncogenes and oncogene-addiction in tumor cells, novel drug targets in tumor biology and a revised mode of target identification are urgently sought after. For one, a profound number of druggable oncogenes driving the majority of cancers in a disease population have now been identified. This means that the drug development efforts to efficiently modulate their function need to be ramped up, both for the druggable and the so far non-druggable oncoproteins, and that more attention to less common oncogenes is required. Drug resistance is a common mechanism by which cancer cells evade the treatment with cytotoxic and targeted drugs, an issue that could be alleviated by synergistic drug combinations targeting multiple cancer-essential cellular processes. It is, therefore, of utmost importance to shed light onto these non-oncogenic processes that tumors are dependent on to find new ways of tackling cancers. Additionally, target-based drug screening, although a successful mode of drug discovery during the past decades, is facing many limitations such as the complexity and heterogeneity of many cancers and the

requirement of a well-described causative target protein, knowledge that is often not available in the case of rare diseases or cancers that share a tumor suppressor gene deficiency.

Tackling some of the challenges faced by current drug target identification approaches was one of the aims of this thesis and the results described in chapter 2 have brought forward new drug targets and drug target candidates in the search for cancer vulnerabilities. On the one hand, a rare hematologic cancer, T-cell prolymphocytic leukemia, was used for a drug repurposing screen which identified BCL-2 inhibition as a useful strategy in the treatment of this disease both *ex vivo* and in the clinics, thereby defining BCL-2 as a new drug target in T-PLL and providing an FDA-approved drug Venetoclax for clinical management of the disease at the same time. Moreover, potential synergistic combination partners of Venetoclax were identified *ex vivo* which might help contribute to a longer-lasting response of T-PLL patients. On the other hand, an RNAi screen in cell lines deficient for subunits of the chromatin remodeling complex BAF brought to light CAF-1 as a novel vulnerability in cancers characterized by mutations in the BAF-complex, representing a non-oncogene addiction of these cells that can serve as a foundation for further drug development efforts. The following chapters will discuss the approaches and the findings in detail.

Ex vivo drug repurposing screen identifies BCL-2 inhibition as treatment strategy in T-cell prolymphocytic leukemia

T-cell prolymphocytic leukemia is a very rare T-lymphoid neoplasm with fatal outcome and usually shows high resistance to conventional chemotherapy. Patients often present with recurrent mutations involving activation of *TCL-1*, a characteristic genetic alteration also used for diagnosis, *ATM*, *MTCP-1*, and *p53* mutations, or trisomies of chromosome 8 together with rearrangement of other chromosomes (Dearden, 2006, 2012). A recent comprehensive mutational analysis including whole-genome sequencing and high-resolution copy number analysis has revealed the genomic landscape in a larger cohort of T-PLL patients (Kiel *et al*, 2014). The study identified gain-of-function mutations in *IL2RG* and the JAK-STAT-pathway, as well as loss-of-function alterations in *EZH2*, *CHECK2*, and *FBXW10*. However, a recent small molecule screen in T-PLL samples could not show any correlation between these newly identified mutations and response to their respective small molecule inhibitors (Andersson *et al*, 2017).

Due to the lack of genomically informed therapy and to identify novel vulnerabilities in this disease, we subjected 86 biopsies of patients with hematologic malignancies to *ex vivo* chemosensitivity testing using a drug library comprised of 106 FDA-approved, well-annotated

anti-cancer drugs as well as small molecules in clinical trials. In parallel, tissue microarrays were generated to correlate drug response to protein expression in these patients. Comparison of drug response was done by calculating the average area under the curve for each drug in T-PLL patients versus non-T-PLL patients. The analysis identified Venetoclax, a specific BCL-2 inhibitor, as the compound with the most differential area under the curve for the two populations tested. Importantly, a recent *ex vivo* drug screen of T-PLL samples also found Venetoclax to be one of the drugs active in T-PLL biopsies, confirming the results of our drug screen in an independent patient cohort (Andersson *et al*, 2017).

Protein expression profiling revealed a positive correlation between drug response and BCL-2 expression level in all patients tested. Moreover, MCL-1 and BCL-X_L expression levels were also positively correlated. Based on these results, Venetoclax was administered to two patients with refractory or relapsed T-PLL after at least two lines of treatment. In both patients, Venetoclax scored as the most potent drug compared to all other molecules in the screen. The two patients responded well during the concentration ramp-up of Venetoclax as evidenced by a decrease in disease-relevant markers such as leukocyte counts, lactate dehydrogenase and beta-2-microglobulin levels, and computer tomography scans of lymphadenopathy as well as ultra sound based examination of splenomegaly. Venetoclax showed significant potency in T-PLL samples from these patients over AML samples *ex vivo*, although not as strong as in CLL samples, the cancer entity that Venetoclax has been approved for. During the course of treatment, both T-PLL patients showed an increase in BCL-2 and BCL-X_L expression as shown by western blot analysis while MCL-1 levels remained unchanged.

Venetoclax (ABT-199) is a BCL-2-specific BH3 mimetic that has recently been approved for the treatment of a subtype of chronic lymphocytic leukemia (CLL) due to overwhelming efficacy in this disease (Roberts *et al*, 2016). It is also the first FDA-approved small molecule inhibitor of a protein-protein interaction that was originally designed through a fragment-based screen. Venetoclax exerts its effect by binding to the hydrophobic BH3 groove of BCL-2, thereby releasing pro-apoptotic proteins BAK and BAX, and inducing apoptosis in cells that rely on constant pro-survival signaling through BCL-2. Reengineering of its predecessor navitoclax (ABT263), a compound that showed promising results in relapsed and refractory CLL but caused thrombocytopenia through co-inhibition of thrombocyte-essential BCL-X_L, resulted in the BCL-2 specificity of Venetoclax (Radha & Raghavan, 2017).

Interestingly, the patient cohort that we screened in chapter 2.1 included one sample from a patient with CLL which showed dramatic sensitivity to Venetoclax, confirming the results that led to Venetoclax approval and underlining the validity of the *ex vivo* screening approach. Moreover, recent clinical studies in acute myeloid leukemias have shown subtype-specific sensitivity to Venetoclax, an observation that is also represented by the variable

response of myeloid leukemia samples to Venetoclax in the *ex vivo* patient cohort (Konopleva *et al*, 2016).

The genetic basis for the overexpression of BCL-2 seen in the majority of CLL patients remains, however, elusive. Human Bcl-2 (B-cell lymphoma 2) was originally described due to its involvement in the t(14;18) chromosome translocation found in the vast majority of follicular lymphomas (FLs), the second most prevalent non-Hodgkin lymphoma in the western hemisphere (Tsujimoto *et al*, 1985; Freedman, 2011). The juxtaposition of Bcl-2 at position 18q21 to the immunoglobulin heavy chain enhancer region (IgH Eμ) located in the 14q32 region creates a Bcl-2/IgH fusion resulting in transcriptional deregulation of Bcl-2 (Korsmeyer, 1992). Unlike other oncogenes that drive cell proliferation, Bcl-2 overexpression causes a constitutive survival signal and impairs the cell's ability to undergo programmed cell death (Vaux *et al*, 1988). Since t(14;18) rearrangements have been reported in non-tumorigenic cells from healthy individuals (Limpens *et al*, 1995), it seems likely that the deregulation of BCL-2 allows clones to persevere until other oncogenic mutations occur. For instance, the overexpression of *c-MYC*, an oncogene generally involved in cell cycle progression, transformation, and proliferation, in combination with cellular stress has been shown to induce apoptosis, which in turn can be inhibited by elevated anti-apoptotic signaling of BCL-2, thereby contributing to uncontrolled cell division rates and cancer development (Bissonnette *et al*, 1992; Limpens *et al*, 1995). Contrary to the findings in FLs, BCL-2 translocations are mostly absent in CLL patients and proposed mechanisms of overexpression such as a decrease in microRNA expression or promoter hypomethylation have still to be elucidated (Dyer *et al*, 1994; Majid *et al*, 2008). Although rearrangements of chromosomes 14 and 18 are often present in patients with T-cell prolymphocytic leukemia, BCL-2 has not been reported to be involved in these translocations either and has not been identified as recurrently mutated so far (Andersson *et al*, 2017).

The network of BCL-2 protein family members is heavily intertwined and comprises a multitude of structurally similar proteins with different functions and tissue-dependent expression patterns. This constitutional intricacy hampers efforts to molecularly dissect the cause-effect relationships of genetic alterations of family members and their functional interaction with other oncogenes. Recently, two other pro-survival signaling proteins of the BCL-2 family, MCL-1 and BCL-X_L, have been shown to be upregulated as a consequence of resistance to Venetoclax-mediated inhibition of BCL-2 in diffuse large B-cell lymphoma cell lines (Choudhary *et al*, 2015). The ability of BCL-2 to sequester pro-apoptotic BIM was reduced in resistant cell lines, while both MCL-1 and BCL-XL were shown to possess an increased potential to bind BIM. This observation could serve as a potential resistance mechanism in T-PLL. The overexpression of BCL-X_L and BCL-2 upon treatment with Venetoclax seen in the treatment of both patients in the screen described in chapter 2.1 could

constitute a compensatory effect that would allow the cells to maintain high pro-survival signaling despite of BCL-2 inhibition. A drug dosage increase might very well counteract the increase in BCL-2 levels, but the possibility of BCL-X_L further compensating for the Venetoclax-mediated inhibition of BCL-2 exists and the status of other additional BCL-2 family proteins and non-BCL-2 proteins supporting this resistance is unknown. For example, Choudhary and colleagues detect concomitant upregulation of AKT while an increase in phosphorylated ERK1/2 has been shown to serve as a protection mechanism against apoptosis induction upon treatment with Venetoclax in follicular lymphoma cell lines (Choudhary *et al*, 2015; Bodo *et al*, 2016). Treatment with PI3K/AKT inhibitors in combination with Venetoclax could restore the resistant phenotype and induce apoptosis in the resistant cell lines.

Similar to the *ex vivo* identification of BCL-2 as a target in T-PLL, we have performed a combinatorial screen to identify drugs that have synergistic effects with Venetoclax in biopsies from T-PLL patients. We have found 7 drugs which enhance the effects of BCL-2 inhibition in these samples, the majority of which has been described as synergistic with Venetoclax in experiments with other hematologic malignancies either *in vivo* or *in vitro*. For instance, 5-azacytidine has been reported to successfully trigger a response in elderly patients with AML in combination with Venetoclax (DiNardo *et al*, 2018). The Bruton tyrosin kinase-specific inhibitor Ibrutinib, the drug that was identified as top hit in our combination screen, is currently being assessed in combination with Venetoclax in clinical studies of relapsed or refractory CLL and preliminary results point into a favorable direction in terms of patient response (NCT02910583). Another kinase inhibitor Imatinib showed substantial cell killing synergies with Venetoclax in a model of CML progenitor cells (Ko *et al*, 2014) On the other hand, we have identified Cisplatin as highly antagonistic in combination with Venetoclax in our *ex vivo* screen. Interestingly, the combination of cisplatin and ABT-737, a predecessor of Venetoclax and pan-BCL-2 family inhibitor, was found to be synergistic in *in vitro* models of non-small cell lung cancer (Kim *et al*, 2017), contrary to our findings. Since ABT-737 also targets BCL-X_L among other BCL-2 family proteins, it could be a reasonable approach to pinpoint the exact BCL-2 protein whose inhibition is necessary for the observed synergism. Moreover, the different physiological background of lung cancer cells versus hematologic clones could serve as an explanation for the effects observed. Cisplatin is mainly used in the treatment of carcinomas and the co-occurrence of T-PLL in patients that already suffer from solid tumors is both very rare and might preclude further treatment due to poor outlook. However, the identification of an approved drug that abolishes the beneficial effects of Venetoclax should raise awareness regarding the study of drug combination treatments and warrants further consideration of the pre-treatment history of individual patients. Taken together, the results from the drug combination screen hint at possible mechanisms by which

T-PLL can be targeted into addition to BCL-2 inhibition but further studies are needed to elucidate the role of these drug targets in combination with Venetoclax and how these proteins are embedded into the BCL-2 family protein network. Moreover, at this point it is not clear whether the combination partners exert their synergistic effects by inhibiting their actual target protein or whether the observed effects are the result of off-targets that could be hit by these drugs.

In summary, the results presented in chapter 2.1 describe the identification of BCL-2 as a vulnerability in T-cell prolymphocytic leukemia using a focused *ex vivo* drug repurposing screen. Protein expression profiling identifies a correlation between response to BCL-2 inhibition and BCL-2 protein expression across the samples where tissue microarray material was available. Based on these results, Venetoclax was administered to two patients with late-stage T-PLL, showing clinical response for the first time in this disease entity upon inhibition of BCL-2. Moreover, upregulation of BCL-X_L and BCL-2 was identified as a potential resistance mechanism of Venetoclax treatment in T-PLL patients and potential drug combination partners were identified, acting synergistically with Venetoclax. These results encourage clinical studies to evaluate the effects of Venetoclax therapy in a larger patient cohort and serve as a starting point for the clinical confirmation of useful drug synergies in combination with Venetoclax.

Histone chaperone CAF-1 is a synthetic lethal target in ARID1A mutant cancers

20% of all cancer patients harbor mutations in the BAF chromatin remodeling complex with ARID1A and SMARCA4 being its most frequently mutated subunits. The majority of mutations found in the BAF complex are loss-of-function mutations, suggesting a *bona fide* tumor suppressor function of many of these subunits. BAF remodels the chromatin by sliding or ejecting histones from DNA using the energy generated by ATP hydrolysis, thereby rendering chromatin more accessible for transcription or repair processes. Additionally, the complex interacts with a multitude of different transcription factors and epigenetic modifiers (Kadoch *et al*, 2013; Pierre & Kadoch, 2017).

Due to its high mutation penetrance in multiple cancer types and the challenges when it comes to targeting the mutated subunits directly, we designed an RNA interference screen to identify synthetic lethal targets in BAF-mutated cancers. The lentiviral shRNA library employed was comprised of 4-5 short hairpin RNA constructs targeting approximately 400 epigenetic modifiers. We screened cell lines deficient for ARID1A or SMARCA4 and compared the hairpin abundance over time to cell lines containing the wildtype BAF complex. Although the BAF mutations in the deficient cell lines were confirmed by next-generation sequencing,

residual ARID1A isoforms were still detectable on a protein level, whereas one of the wildtype cell lines, NCI-H146, showed a considerably lower amount of ARID1A. We tested various ARID1A antibodies but neither of them detected one single band at the expected band size of 250kDa. This could either mean that none of the antibodies reacts specifically with the 250kDa protein or that more than the known isoforms exist in a cell line specific manner, potentially with various post-translational modifications. Moreover, conclusions about the remaining activity of the complex are difficult to make since most of the chromatin remodeling assays described in the literature are performed *in vitro* which might hamper the correlation to the physiological conditions (Chen *et al*, 2014). Nevertheless, neither complete loss of ARID1A nor homozygous mutations are commonly seen in human cancers and the cell lines deficient for ARID1A in the screen can therefore be regarded as representative (Wu & Roberts, 2013).

The algorithm used for genes to be regarded as synthetic lethal candidates required at least two hairpins targeting this gene in two or more deficient cell lines, leading to a hairpin abundance-based Z-score of less than -1, while sparing both BAF-wildtype cell lines. Some of the published synthetic lethal interaction partners were also part of the shRNA library, such as EZH2, ARID1B, and the recently found HDAC6 vulnerability in ARID1A deficient cells and SMARCA2 in SMARCA4 deficient cells, respectively, but closely missed the threshold of the selection criteria (Helming *et al*, 2014; Hoffman *et al*, 2014; Kim *et al*, 2015; Bitler *et al*, 2015, 2017). The reason for this might be a less-than-optimal infection efficiency in the respective cell lines for some of these constructs. The potential hit genes were subject to colony formation assays in which a subset of cell lines was infected with the same lentivirus used in the screen. This assay validated CHAF1A and CHAF1B, two subunits of the CAF-1 histone chaperone complex, as synthetic lethal targets in ARID1A and SMARCA4 deficient cell lines.

The histone chaperone CAF-1 (chromatin assembly factor 1) was identified in 1989 as a heterotrimeric complex constituted of three protein subunits which facilitate the incorporation of histones into newly formed DNA during replication and DNA repair (Smith & Stillman, 1989). The main function of CAF-1 consists in delivering histone H3/H4 dimers to the replication fork at newly synthesized DNA during S-phase. The three subunits were initially named after their molecular weight: p150 (CHAF1A), p60 (CHAF1B), and the highly abundant p48 (RbAp48) make up the CAF-1 complex in a 1:1:1 stoichiometry. Purification analysis revealed that the complex often contains newly synthesized dimers of histones H3/H4 in their acetylated form and later studies found that CAF-1 preferentially binds the replication-dependent H3 variant H3.1, produced mainly during S-phase (Verreault *et al*, 1996; Volk & Crispino, 2015).

CHAF1A, the largest subunit of the CAF-1 complex, contains two distinct proliferating cell nuclear antigen (PCNA) interacting peptide regions (PIPs) of which only PIP2 binds to PCNA with a high affinity *in vivo*. This interaction allows the complex to target the replication fork directly through interaction with the sliding clamp that is formed by PCNA which serves

as a scaffold for proteins of the replication machinery (Rolef Ben-Shahar *et al*, 2009). CHAF1A can directly interact with new acetylated histones via two protein domains, one that is rich in lysine, glutamic acid, and arginine residues (KER domain) and another one that is enriched for glutamic acid and aspartic acid (ED domain). In addition to the S-phase-associated functions of CHAF1A, it also plays an important role in DNA damage repair during interphase. Depending on the type of damage, the subunit either acts in concert with PCNA during nucleotide excision repair (NER) or via interaction with the DNA-dependent Protein Kinase (DNA-PK) complex to repair double strand breaks (DSBs) (Moggs *et al*, 2000). Moreover, the protein sequence of CHAF1A includes an HP1 (heterochromatin-binding protein 1) domain and protein domains that interact with SETDB1, an H3K9 methyltransferase. The interaction with HP1 was confirmed as an important factor in heterochromatin maintenance, thereby constituting another essential role of CAF-1 besides DNA replication and repair (Cheloufi & Hochedlinger, 2017).

The medium-sized CHAF1B subunit contains 7x WD repeats, two B-domain-like motifs for interaction with the H3/H4 histone chaperone ASF-1, as well as a PEST (proline, glutamic acid, serine, threonine rich) domain (Volk & Crispino, 2015). CHAF1B can be phosphorylated and its phosphorylation status correlates with subcellular localization. The protein kinases responsible for this posttranslational modification are Cyclin-Dependent Kinases (CDKs) and DNA-PK, which has been reported in CHAF1A-mediated double-strand break repair (Marheineke & Krude, 1998). Interestingly, *CHAF1B* activating mutations have been implicated in Down syndrome, potentially causing defects in nucleosome assembly (Katsanis & Fisher, 1996). Moreover, *CHAF1B* alterations in cancer are exclusively characterized as missense or silent mutations while no truncations, deletions, or insertions have been identified (Volk & Crispino, 2015). Expression levels were shown to accurately predict the occurrence of metastases in salivary gland tumors or prostatic cancers, while other CAF-1 components were not overexpressed in these samples (Staibano *et al*, 2009, 2011). These findings point towards a CAF-1-independent role of CHAF1B in the development of metastases. In addition, CHAF1B expression serves as a prognostic marker in endometrial, cervical, renal, and breast carcinomas where it correlates with poor clinical outcome (Polo *et al*, 2010).

The smallest subunit of CAF-1, p48, is the most abundant of the three subunits, with only a small percentage being associated with the complex. Additional roles have been suggested, such as regulation of the repressive H3K27me3 histone mark deposition by PRC2 via its interaction with the histone H3/H4 dimers (Vizán *et al*, 2015). Interestingly, p48 is involved in histone deacetylase processes via direct interaction with the Mi-2/NuRD, a protein complex that has both deacetylase and ATP-dependent chromatin remodeling activity.

Knockdown experiments have shown a dramatic influence of CAF-1 on cellular plasticity during development and reprogramming. For instance, in an RNA interference

screen with the goal to identify chromatin-associated factors that could safeguard somatic cell identity during transcription factor-mediated reprogramming of murine fibroblasts into induced pluripotent stem cells, *CHAF1A* and *CHAF1B* were found as the top hits. Knockdown of these subunits resulted in a higher chromatin accessibility and a reduction in heterochromatic domains, thereby increasing the activity of pluripotency-specific genes (Cheloufi *et al*, 2015). So far, no direct interaction between BAF and CAF-1 has been reported although the overlap in functional areas such as modulation of chromatin accessibility, DNA replication and repair, as well as nucleosome assembly and exchange are undeniable.

The diverse genetic background of the cell lines used in the screen might have compromised some of the *CHAF1A* and *CHAF1B*-targeted hairpin constructs and stronger effects upon depletion of CAF-1 could have potentially been overseen. To confirm CAF-1 as a vulnerability in BAF-deficient cells and to circumvent the challenges of genetic heterogeneity, we employed an isogenic HAP1 cell line model in which the only difference between the cell lines was the lack of ARID1A in the knockout clones. A GFP competition assay in which GFP^+ wildtype cells and GFP^- HAP1::ARID1A cells were mixed, infected with individual shRNA's, selected and propagated for several days, showed a decrease in the abundance of *ARID1A* knockout cells. Although western blot analysis showed a decrease in CHAF1A and CHAF1B abundance upon knockdown, confirming the on-target activity of the constructs, the magnitude of *ARID1A* knockout cell killing in the GFP competition assay did not correlate with the protein decrease by the respective hairpins. One reasons for that could be a tight dosage requirement of the CAF-1 complex within the cell so that a too efficient knockdown of the protein might lead to less pronounced differential cell killing. *CHAF1A* and *CHAF1B* are essential genes in HAP1 and cell death of HAP1 wildtype cells upon knockdown of either of the two CAF-1 subunits was noticed, but to a much lesser extent as in the *ARID1A* knockouts. This could mean that only a tight therapeutic window in these cells would be available, similar to FDA-approved drugs that target essential cellular factors such as the proteasome. Another explanation for the lack of correlation between cell killing and knockdown efficiency could be the knockdown of other proteins besides CAF-1.

To exclude any such off-target effects, we used siRNA constructs that were chemically modified to yield a low off-target efficiency in the same assay and a rescue experiment in which the endogenous target site of one of the hairpins was altered. The siRNA knockdown of CAF-1 with a pool of 4 highly target-specific siRNA constructs showed the same *ARID1A* knockout-specific phenotype in the GFP competition assay. Knockdown of *CHAF1B* resulted in a decrease of CHAF1A in both cell lines, probably through exposure of the CHAF1A PEST domain, leading to rapid protein degradation (Volk & Crispino, 2015). To fully rule out the possibility of any off-target effects and indubitably confirm CAF-1 as an ARID1A-specific vulnerability, we generated an inducible CHAF1A knockdown system in HAP1 wildtype and

HAP1::ARID1A cells which we could rescue through silent genomic alterations in the respective shRNA target sequence. The observed increase in *ARID1A* knockout cell abundance is due to the overall toxicity of shCHAF1A_125 as described above via targeting an essential cellular function. Infecting the resistant and wildtype cell lines with another hairpin, shCHAF1A_126, resulted in the expected cell death phenotype upon knockdown induction, as seen in the previous experiments. Having employed various constructs and systems to target multiple regions within the *CHAF1A* and *CHAF1B* locus, as well as engineering a cell line that is resistant to one of the CHAF1A hairpins but not to a second one ultimately emphasizes the on-target effects of the hairpins used and confirms CAF-1 as a synthetic lethal target in an ARID1A null landscape.

Mechanistically, the knockout of *ARID1A* in HAP1 cells results in the differential regulation of more than 600 genes. The vast majority of these genes are annotated with the functional terms associated with PRC2. These findings emphasize the reported synthetic lethality between EZH2, the methyltransferase subunit of PRC2, and ARID1A deficiencies (Bitler *et al*, 2015; Kim *et al*, 2015). The examination of transcriptional CAF-1 knockdown effects in *ARID1A* mutant cells did not point towards a deregulation of specific genes or gene families that could be responsible for the observed cell death phenotype. On a global level, however, we could show that the majority of genes that were deregulated upon loss of ARID1A were expressed at a level closer to the wildtype condition when *CHAF1A* or *CHAF1B* was knocked down on top, implying a genetic rescue of *ARID1A knockout* effects through depletion of CAF-1. In accordance with these findings, ATAC-seq analysis shows similar effects when examining the changes in nucleosome positioning (Schep *et al*, 2015). Loss of ARID1A results in a shift of fragment length towards longer DNA fragments shielded by histones, suggesting a lower overall accessibility of DNA, similar to the expected effects of a BAF chromatin remodeling deficiency. Knockdown of either *CHAF1A* or *CHAF1B* has the opposite effect, enriching the density of shorter DNA fragments in both wildtype and *ARID1A* knockout cells. A differential density map depicts the overall changes compared to the wildtype condition where fragment lengths of 147 bp and ~160 bp are enriched over the wildtype when knocking down CAF-1 in *ARID1A* knockout cells. These fragment lengths correspond to the usual length of DNA wrapped around the histone octamers (147 bp) and a bigger fraction that could, for instance, be explained by an increase in linker histone H1 incorporation into the nucleosome, forming what is called the “chromatosome” (Fyodorov *et al*, 2017). From the results of transcriptional profiling and chromatin accessibility analysis however, a clear link between cell death upon knockdown of the CAF-1 complex in *ARID1A* knockout cells could not be established and further research is warranted regarding the roles of chromatin dynamics upon BAF deficiency and depletion of histone chaperones.

In a final step, we employed a drug screening library with >2,000 well annotated or highly toxic compounds with the goal to identify synergistic partners of CHAF1A knockdown in the isogenic cell line. Using the inducible isogenic HAP1 cell line pair discussed above we could identify the androgen receptor agonists testosterone and 19-nortestosterone as specifically acting on *ARID1A* knockout cells upon induction of *CHAF1A* knockdown. The effect was validated in manual dose-response curves revealing a IC_{50} of approximately 1 µM for both molecules and in a GFP competition assay using two inducible hairpins against CHAF1A. The BAF complex has been reported to govern androgen-dependent expression of androgen receptor target genes and that BAF-deficiency abrogates this signaling cascade (Marshall *et al*, 2003). The only link between *CHAF1A* expression and testosterone in the literature was described as a general transcriptional upregulation of *CHAF1A* upon treatment with testosterone in a LNCaP cells, a prostate cancer cell line, at nanomolar concentrations (Wang *et al*, 2011). The high dose required for *ARID1A* knockout plus shCHAF1A-specific cell death induction, however, speaks against an androgen receptor-mediated mechanism. Dihydrotestosterone has been shown to induce DNA damage response pathways under oxidative stress or by application of polyphenols in prostate cancer cell lines at similar concentrations used in our assay, as evidenced by an increase in γH2AX levels (Ide *et al*, 2011, 2012). We could not detect an increased γH2AX intensity, neither upon induction of *CHAF1A* knockdown nor upon testosterone treatment (data not shown). This might be due to γH2AX-independent DNA repair mechanisms, a narrow time window of γH2AX presence, or the absence of DNA damage at the effective concentration used in HAP1 cells.

In summary, we could identify the histone chaperone complex CAF-1 as a synthetic lethal vulnerability in concert with ARID1A deficiency. This non-oncogene addiction of the BAF complex was found in multiple cancer cell lines and has been confirmed in an isogenic cell line model. Additional experiments in BAF-wildtype NCI-H2122 lung cancer cells point towards a similar trend when ARID1A is lost via CRISPR -mediated knockout generation (unpublished master thesis of Melanie Hinkel), which could suggest a hard-wired relationship between BAF and CAF-1. Transcriptional profiling and nucleosome positioning assays did not reveal single genes or gene families responsible for the observed cell death phenotype in ARID1A deficient cells. We could, however, identify a reversal of deregulated genes upon knockdown of CAF-1. The finding that testosterone enhances the *ARID1A knockout*-specific cell death upon CAF-1 knockdown together with literature reports about testosterone-induced DNA damage, as well as the involvement of CAF-1 and BAF in DNA repair hints at an involvement of DNA damage and response pathways in this striking phenotype. It is tempting to suggest that an increased rate of DNA damage can be compensated when either BAF or CAF-1 are fully functional but leads to cell death when both complexes are impaired. Further research is required to unravel

the mechanism behind the non-oncogene addiction described in this thesis. Nevertheless, the identification of CAF-1 as a synthetic lethal strategy to tackle *ARID1A*-mutated cancers presents a potential basis for drug development efforts towards a more efficient treatment of BAF-deficient tumors.

3.2 Conclusion and future prospects

This thesis aimed at identifying novel cancer vulnerabilities using focused chemical and genetic screening approaches. The results presented in the first part describe the application of an *ex vivo* chemosensitivity screen using FDA-approved drugs and small molecules in clinical development to shed light onto the cellular dependencies of these cancer cells and exploit drug repurposing opportunities in the treatment of T-cell prolymphocytic leukemia. The successful identification of the BCL-2-inhibitor Venetoclax constitutes a highly relevant finding in the context of a disease that lacks effective treatment options. Inhibition of BCL-2 will hardly be a curative treatment for T-PLL patients. Recognizing the importance of this protein for the pathobiology, however, will contribute to a more systematic treatment in the future. Drug combination partners for Venetoclax are urgently sought after and further *ex vivo* screens hve identified 6 drugs that act synergistically with Venetoclax. Given the increasing knowledge of the genetic and epigenetic landscape of T-PLL together with the *ex vivo* response of patient material with single drugs or drug combinations can overcome the obstacles set by the rarity of this disease and, in the long run, hopefully result in a long-lasting treatment response of patients suffering from one of the most aggressive forms of leukemia.

In the second part of this thesis, histone chaperone complex CAF-1 was identified as a vulnerability in cancer cells deficient for the BAF-subunit ARID1A using an epigenome-focused RNA interference screen. The relevance of this finding is emphasized by the high occurrence of BAF-subunit loss-of-function mutations in many different cancer entities, affecting 20% of all cancer patients. Despite the confirmation of this novel non-oncogene addiction, many questions remain and will need to be addressed by further research. Why do *ARID1A* knockout cells die specifically when they lose CAF-1 subunits? Is this effect hard-wired or is it inherent to only a sub-population of cancers? Will there be a possibility to design a small molecule inhibitor for this histone chaperon, given its extended interaction surface with other CAF-1 complex members and histones? Can fragment-based screening facilitate this process, as it did in the case of Venetoclax? Will the effects of CAF-1 inhibition by small molecules be similar to the reduction of its protein levels, i.e. will CAF-1 structure play a role in the observed synthetic lethality? Could target-based degradation be a possibility to deplete

enough CAF-1 from the tumor cells? Is the remaining structure of the BAF complex predictive of CAF-1 vulnerability? Based on the findings described in this thesis further studies are hopefully going to shed light onto some of these questions, thereby contributing to the generation of novel treatment strategies for tumor suppressor mutations affecting the BAF chromatin remodeler.

4. Material & Methods

Material and methods used in the published part of chapter 2.1 can be found in the original publication. Other material and methods used in the unpublished results sections are described below.

Synergy screen in T-PLL patient samples

Drug effects were calculated as percent of control (POC) in regard to the effects of the negative control (DMSO) and positive control (10 µM Bortezomib). Patients with noisy Z-sores were excluded from the analysis. Drug synergy effects were calculated as deviation from Bliss. The bliss independence model allows the estimation of drug combinations where the effect C = A + B – A x B with A being the fractional inhibition of drug A and B being the fractional inhibition of drug B (Bliss, 1939; Licciardello *et al*, 2017). The results of the drug combination screen were then compared to the respective Bliss score and the deviation of each combination form the score was plotted on a heatmap. Positive deviations denote synergistic effects while negative deviations denote antagonistic effects. Concentrations used for each compound are shown below.

Table 1 Drug concentrations used in T-PLL combination screen.

Compound Name	CP1 (µM)	CP2 (µM)	CP3 (µM)	CP4 (µM)	CP5 (µM)
5-Azacytidine	27	9	3	1	0
6-Mercaptopurine	13.5	4.5	1.5	0.5	0
Venetoclax	0.2	0.067	0.022	0.0074	0
Alitretinoin	27	9	3	1	0
Bendamustine	27	9	3	1	0
Bortezomib	0.00803	0.00268	0.000893	0.000298	0
Cisplatin	27	9	3	1	0
Hydroxyurea	27	9	3	1	0
Ibrutinib	13.5	4.5	1.5	0.5	0
Idelalisib	13.5	4.5	1.5	0.5	0
Imatinib	17.16795	5.72265	1.90755	0.63585	0
Lenalidomide	5.624775	1.874925	0.624975	0.208325	0
Pentostatin	19.8315	6.6105	2.2035	0.7345	0
Prelone	1.586925	0.528975	0.176325	0.0588	0
Vincristine	2.997	0.999	0.333	0.111	0

Cell lines and culture conditions

Cell lines for RNAi screen were sequenced and kindly provided by Boehringer Ingelheim as part of the Christian Doppler Laboratory. HAP1 wildtype (C631) and HAP1::ARID1A (HZGHC000618c010) cells were obtained from Horizon Genomics. Culture media used for individual cell lines are described below. All media were supplemented with 1% penicillin-streptomycin (Sigma Aldrich). Cell lines carrying an inducible knockdown construct were cultured using tetracycline-free fetal bovine serum (FBS, Biowest/VWR). For all other cell lines, heat-inactivated standard fetal bovine serum (Thermo Fisher Scientific) was used as a supplement at the indicated percentage. Cells were incubated at 37°C and 5% CO_2.

Table 2 Cell lines and culture conditions.

Cell line	Medium	Provider
NCI-H146	RPMI + 10% FBS	Thermo Fisher Scientific
HCC827	RPMI + 15% FBS	Thermo Fisher Scientific
RL95-2	DMEM/F12 + 10% FBS	Thermo Fisher Scientific
SK-UT-1	EMEM + 10% FBS	Thermo Fisher Scientific
SKOV-3	McCoy's 5A + 10% FBS	Thermo Fisher Scientific
NCI-H1568	RPMI + 10% FBS	Thermo Fisher Scientific
NCI-H522	RPMI + 10% FBS	Thermo Fisher Scientific
A549	F-12K + 10% FBS	Thermo Fisher Scientific
HCC-366	RPMI + 10% FBS	Thermo Fisher Scientific
HAP1	IMDM + 10% FBS	Thermo Fisher Scientific
HAP1::ARID1A	IMDM + 10% FBS	Thermo Fisher Scientific
293T	DMEM + 10%FBS	Thermo Fisher Scientific

Immunoblotting

Cells were harvested, washed 1x with phosphate buffered saline (PBS, Thermo Fisher Scientific) and lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche) at 4°C. The soluble protein fraction was transferred to a new tube and protein concentration was measured using Bradford reagent (VWR) and a bovine serum albumin (BSA, Sigma Aldrich) standard curve. 50 µg lysate were loaded onto a 7% or 10% sodium dodecyl sulfate polyacrylamide gel depending on the protein size of interest and resolved at 125 V and 35 mA for 2 hours. The proteins were transferred onto a nitrocellulose blotting membrane (Amersham Protran, Merck) at 30 V and 200 mA for 90 minutes. The membrane was blocked with 5% blocking grade blocker (Biorad) in TRIS-buffered saline supplemented with 0.05% Tween-20

(TBS-T) for 1 hour at room temperature, incubated with the primary antibody over night at 4°C in 5% blocking grade blocker (1:1000), washed 3x 5 minutes with TBS-T, incubated with the horse radish peroxidase-conjugated secondary antibody (1:10000, Jackson Immuno Research) in TBS-T for 1 hour at room temperature and washed again 3x 5 minutes with TBS-T. Membranes were covered in Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) for 1 minute and exposed to Amersham High Performance Chemiluminescence films (VWR) in the dark before development. Primary antibodies used were obtained from the sources below.

Table 3 Antibodies used for immunoblotting.

Target	Provider
ARID1A (ab182560)	Abcam
SMARCA4 (sc17796)	Santa Cruz
CHAF1A (ab126625)	Abcam
CHAF1B (ab8133)	Abcam
α-Tubulin (ab7291)	Abcam
β-Actin (ab8224)	Abcam

Epigenome-focused library and RNAi screen

The lentiviral library used in the screen was produced at Sigma Aldrich and is based on the pLKO vector including a Puromycin resistance cassette as previously described (Licciardello *et al*, 2015). Virus titer and evaluation of required puroymcin concentration was performed for each cell line to ensure equal multiplicities of infection. 2 x 10^6 cells per cell line were infected with pooled virus at an MOI of 1 in quadruplicate with 8 µg/ml of polybrene (Santa Cruz), selected with puromycin (Thermo Fisher Scientific) for 3 days and kept in culture for 14 days in total. Time points were taken on day 1 and day 14 post infection. Genomic DNA was then isolated using the Wizard Genomic DNA Purification Kit (Promega), concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Outer PCR was performed using ExTaq polymerase (Takara) and 4 µg of DNA per sample, followed by inner PCR to attach the sequencing barcodes for each sample. Samples were then purified using a QIAquick PCR Purification Kit (Qiagen) and submitted to the Biomedical Sequencing Facility at CeMM for 50 bp single-end sequencing. Differential abundance of hairpins between last and first time point was analyzed.

For the RNAi screen, sequenced barcodes correlating to different samples (cell line, time point, replicate) and the abundance of hairpin sequences in these samples were analyzed

and samples with low read number or poor correlation between the four replicates per time point and cell line ($r \leq 0.72$) were discarded. Hairpin read numbers of less than 100 reads were set at 100 to avoid extreme values. Read numbers were normalized to the barcode and respective vector carrying this hairpin (different vector versions were present in the library, all based on pLKO). Z-scores were calculated for each hairpin in every cell line comparing the first and last time point in the screen.

shRNA and siRNA constructs for target validation

Small hairpin RNA oligos were ordered from Sigma Aldrich and cloned into a pLKO vector carrying a Puromycin resistance cassette. Plasmids were transfected into 293T cells by calcium phosphate transfection together with a pCMV-dR8.91 packaging plasmid and a pCMV-VSV-G envelope plasmid (a gift from Bob Weinberg, Addgene plasmid #8454) for lentivirus production. Lentivirus was collected on day 2 and 3 post transfection and kept at -80°C and used for infection in combination with 8 µg/ml polybrene (Santa Cruz). Cell lines were selected with Puromycin for expression of lentiviral vector. Inducible constructs were ordered from Dharmacon as SMARTvector Inducible Lentiviral shRNA constructs and used for lentivirus production as described above.

Table 4 shRNA constructs and target sequences for CHAF1A knockdown.

Gene / Construct	Target Sequence
CHAF1A_1	ACAAGCCCGTCTGCCGTTTAA
CHAF1A_2	AGAAGAAGAGAAGCGCATTAA
CHAF1A_3	TAACTAAGAAATTCGTCAAAG
CHAF1A_125 (inducible)	GAAGAAGAGAAACGGTTAA
CHAF1A_126 (inducible)	GCGGCGAGTTCTCCTTCTT
CHAF1A_127 (inducible)	GATCAGCAGGGGTTGTTGA

Table 5 shRNA constructs and target sequences for CHAF1B knockdown.

Gene / Construct	Target Sequence
CHAF1B_1	GCGAGTATACAGTATACAGAA
CHAF1B_2	CGTCATACCAAAGCCGTCAAT
CHAF1B_3	CATCCTATTGTGGAAGGTGAA
CHAF1B_4	GCTGTTGTATTCAGTATCCAT

Pools of 4 siRNA constructs per target gene were ordered from Dharmacon and resuspended in H_2O to a final concentration of 10 µM. siRNA transfection was carried out using Dharmafect reagent #1 (Dharmacon) according to the manufacturer's protocol. Medium was replaced on the next day.

Table 6 siRNA pools and target sequences.

Gene / siRNA pool	Target Sequence
pooled siCHAF1A	CCACAGCCAUGGAUUGCAA
	GGGCAAGCAGCUCAAGUUA
	GACAUAGACUUUAGACCGA
	AAACAACUGUCAUGUGGGU
pooled siCHAF1B	GCAACUGAUGGGAAUUUAA
	AAACAGGUGUGGAGCUGAU
	GAAGCUACCGGAUGUUUCA
	GGACGGUUACUGCUCAUUU

GFP competition assay

HAP1 wildtype cells were infected with a pLenti-CMV-GFP-Neo construct (a gift from Eric Campeau & Paul Kaufman, Addgene plasmid #17447) and sorted as a pure population. HAP1 wildtype (GFP^+) cells and *ARID1A* knockout cells (GFP^-) were mixed, equally distributed onto wells of a 6-well plate and incubated overnight. On the next day, cell mixes were either infected with lentivirus containing constitutively active shRNA constructs against *CHAF1A*, *CHAF1B*, or a non-targeting control, or transfected with siRNA's as described above. In the case of shRNA's, cells were selected using 2 µg/ml Puromycin. Abundance of GFP positive and negative cell population was measured using fluorescence-activated cell sorting (FACS) and compared to the control condition. All experiments were performed in duplicate or triplicate.

For inducible constructs, wildtype (GFP^+) and knockout (GFP^-) cells were infected with the inducible shRNA vectors carrying a RFP (targeting and non-targeting control), induced with 1 µg/ml doxycycline (Sigma Aldrich) for 24h and GFP^+/RFP^+ wildtype cells as well as RFP^+ knockout cells were sorted into pure fractions. Cells were then incubated, counted, mixed, and equally distributed onto wells of a 6-well plate and incubated overnight. On the next day, cells were induced with 1 µg/ml doxycycline and abundance of GFP positive and negative cells was measured over time using FACS. Doxycycline was renewed every 2-3 days.

Similarly, the GFP competition assay for drug effect validation was carried out in the presence of 1 µM testosterone (Sigma Aldrich), 19-nortestosterone (Sigma Aldrich), or DMSO (Sigma Aldrich) and 1 µg/ml doxycycline. Medium containing doxycycline and compounds was renewed every 2-3 days.

Homologous recombination of endogenous CHAF1A locus

HAP1::ARID1A cells were transfected with a lentiCRISPRv2 plasmid (a gift from Feng Zhang, Addgene plasmid # 52961) carrying a Cas9 endonuclease and a guide RNA which directs the endonuclease to cut at the shCHAF1A_125 target site. A 200 bp DNA-template (Integrated DNA Technologies) containing the desired point mutations was co-transfected, as was a pLV-mCherry plasmid (a gift from Pantelis Tsoulfas, Addgene plasmid # 36084) to control for successful transfection. $mCherry^+$ cells were then sorted as single clones into 96-well plates, incubated until the colonies grew out and one sample per clone was subject to PCR amplifications (Terra^{TM} PCR Direct Polymerase Mix, Takara) with primers specific for the point-mutated locus. Cells that showed a homologous recombination site on at least one allele were again used for PCR with wildtype primers to determine homozygosity or heterozygosity of inserts. Finally, the recombined locus was amplified with a primer pair spanning the recombined region and sent for Sanger sequencing to confirm successful recombination. All oligos except the guide RNA were obtained from Sigma Aldrich. Reverse primer bound downstream of the 200 bp DNA template region.

Table 7 Oligos used for homologous recombination.

Oligo	Sequence
guide RNA (CHAF1A Exon 6)	CACCGGAAAAAGGAAGAAGAGAAA
Wildtype primer forward (target region of shRNA)	GCTAAACTTGAGGAAAAAAGGAAAAA GGAAGAAGAGAAACGGTTA
Recombined primer forward (target region of shRNA)	TTGAGGAAAAAAGGAAAAAGGAGGAG GAAAAGCGATTG
Reverse primer	AGCACTGGGAAGAAGCAACAAGAACGCA
Sanger forward primer	CCTTATACTAGACATGAAAATAACTTATATT CATTTAATGTAAAATGAAATACTTGCCATAT ACTAACAGTTGA
Point-mutated template DNA	TGCTGCGGTCCCTTCCAGAGCCAAAGCAAAG AGTCGGCTGAAATGTCATTTGCTGTCTCACAG GGCTAAACTTGAGGAAAAAAGGAAAAAGGAGG AGGAAAAGCGATTGAGAGAAGAAGAGAAGGTA GAGTGTTTCCCACAGAGCTTCCCCGTCCCAGC CCGTTGGAGAAGCAGATGCCCAAAGTGGAATT CTTGCCACA

Transcriptional and chromatin accessibility profiling

HAP1 cells for transcriptional and chromatin accessibility profiling were infected with hairpins against *CHAF1A*, *CHAF1B*, or a non-targeting control hairpin in duplicate and selected with 2 µg/ml Puromycin for 48 hours. 3 days post infection, cells were harvested, washed 1x in PBS.

For transcriptional profiling, one part of the sample was used for RNA isolation with a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and RNA was submitted for library preparation and sequencing to the Biomedical Sequencing Facility at CeMM. RNA-seq reads were aligned to the human genome using TopHat software and gene expression profiling and comparisons was carried out by Cufflinks software at the Biomedical Sequencing Facility. Gene set enrichment analysis was done with the EnrichR package for R software (Chen *et al*, 2013) using the entirety of databases present on the server.

To determine chromatin accessibility and nucleosome positioning effects, ATAC-seq was performed as previously described (Buenrostro *et al*, 2013; Rendeiro *et al*, 2016). Cells were counted and $5x10^4$ cells of each sample were lysed with ATAC-seq lysis buffer for 10 minutes at 4°C. Samples were all prepared in duplicate. Cells were centrifuged, washed in $MgCl_2$ buffer once and incubated in the transposase mix (Illumina) for 30 minutes at 37°C.

DNA was purified using the MinElute PCR Purification Kit (Qiagen) and part of the eluted DNA was employed in a qPCR reaction to determine amplification cycle number. SPRI size selection was used to keep fragment size below 1.2 kb. Samples were sequenced at the Biomedical Sequencing Facility (BSF) at CeMM.

2k library and drug screening in HAP1 cells

The 2k library consists of more than $2x10^3$ small molecules that are either highly annotated or have been dramatically toxic in previous screens with other cell lines. In a first round, the compounds have been screened at a single concentration (mostly 10 µM final concentration). HAP1 wildtype cells and HAP1::ARID1A cells, both either with integrated inducible shControl or shCHAF1A_125 constructs were induced with 1 µg/ml doxycycline for 3 days and seeded onto the 384-well drug plates using a Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific) in the presence of fresh doxycycline. The plates were incubated at 37°C and 5% CO_2 for 72 hours and CellTiter-Glo Luminescent Cell Viability assay was used to measure cell viability according to the manufacturer's protocol. DMSO was used as a negative control, 10 µM final concentration of Bortezomib was used as a positive control. Drug effects were assessed as percent viability of control (POC). Steady RFP expression of all four cell lines was confirmed at 3 and 6 days post induction, corresponding to the time of seeding and time of readout with an Operetta High-Content Imaging System (Perkin Elmer). Multi-point dose response curves for validation of drug hits were done in manual dilution series in 96-well plates but otherwise in the same fashion as the large-scale screen.

Work with viral particles and patient samples

All work involving lentivirus and patient material was carried out in an environment classified as biosafety level 2 according to standard operating procedures and institutional guidelines at the Center for Molecular Medicine (CeMM).

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Doctor of Philosophy (PhD)

Vienna University of Economics and Business | Vienna, Austria — *03/2007 – 02/2016*
Bachelor of Science (BSc), Business Administration

Harvard Medical School | Boston, MA, USA — *07/2010 – 07/2011*
Diploma thesis, laboratory of Benjamin Ebert

University of Vienna | Vienna, Austria — *10/2005 – 08/2012*
Magister rerum naturalium, Molecular Biology

Tassilo-Gymnasium Simbach | Simbach am Inn, Germany — *09/1997 – 06/2005*
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Professional Experience

CeMM Center for Molecular Medicine Predoctoral Fellow | Vienna, Austria — *09/2012 – present*

Harvard Medical School, Ben Ebert lab Diploma Student | Boston, MA, USA — *07/2010 – 07/2011*

Bosch Automotive Aftermarket Intern Business Development | Lima, Peru — *02/2009*

Boehringer Ingelheim, Frank Hilberg lab Intern | Vienna, Austria — *11/2009 – 12/2009*

Harvard Medical School, Ben Ebert lab Intern | Boston, MA, USA — *08/2009 – 10/2009*

Max F. Perutz Laboratories, Tim Skern lab Intern | Vienna, Austria — *03/2009 – 04/2009*

Medical University of Vienna, VIETAC lab Intern | Vienna, Austria — *08/2008 – 09/2008*

Achievements & Awards

- Oral presentation at American Society of Hematology Conference, Atlanta, USA (2017)
- American Society of Hematology “Abstract Achievement Award” (2017)
- Two “Keystone Future of Science” fellowships (2015 & 2016)
- Performance fellowship University of Vienna (2010)

Publications

- Boidol et al. "*First in human response of BCL-2 inhibitor Venetoclax in T-cell prolymphocytic leukemia"* **Blood** (2017)
- Licciardello et al. *"A combinatorial screen of the CLOUD uncovers a synergy targeting the androgen receptor"* **Nature Chemical Biology** (2017)
- Valent et al. *"TKI rotation-induced persistent deep molecular response in multi-resistant blast crisis of Ph+ CML"* **Oncotarget** (2017)
- Ajore et al. *"Deletion of ribosomal protein genes is a common vulnerability in human cancer, especially in concert with TP53 mutations"* **EMBO Molecular Medicine** (2017)
- Englinger et al. *"Acquired nintedanib resistance in FGFR1-driven small cell lung cancer: role of endothelin-A receptor-activated ABCB1 expression"* **Oncotarget** (2016)
- Licciardello et al. *"NOTCH1 activation in breast cancer confers sensitivity to inhibition of SUMOylation"* **Oncogene** (2015)
- Herzog et al. *"A strand-specific switch in noncoding transcription switches the function of a Polycomb/Trithorax response element"* **Nature Genetics** (2014)

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- German (mother tongue), English (fluent), Spanish (beginner), Portuguese (beginner)
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